REMARKS

Claims 1-11 and 12-27 are pending in the application. Claims 12-27 are withdrawn from consideration as being drawn to a non-elected invention. Claims 3-4 and 8 have been canceled without prejudice or disclaimer. Claims 1, 2, 5, 6, 7, 9 and 11 have been amended to better clarify what Applicants regard as the invention. New claims 28-33 have been added for consideration. Support for the amendments can be found throughout the specification, but particularly in original claims 3-4, and 8. Further support for the amended claims can be found throughout the specification and particularly on page 3, paragraphs [0008]-[0009]; on page 5, paragraph [0019]; on page 10, paragraph [0041]; on page 28, paragraphs [0094] and [0098]; and on page 31, paragraph [0105]. No new matter has been added by way of this amendment. Thus, as a result of the foregoing amendment, and if the new claims are entered for consideration, claims 1, 2, 5-7, 9-11 and 28-33 are under consideration. Reconsideration of this application is respectfully requested.

Rejection under 35 U.S.C. §112, first paragraph

By way of this Action, the Examiner has rejected claims 1–11 under 35 U.S.C. §112, first paragraph, for failure to comply with the written description requirement. In particular, the Examiner alleges that claim 1 recites an endopeptidase enzyme having a pH optimum between 7.5 and 10, an apparent MW of about 14 kDa and claims 2 and 7-10 are directed to additional properties of the claimed endopeptidases while claims 3-6 recite genera and species of microorganisms that produce the claimed enzyme.

However, the Examiner further alleges that the as-filed specification provides a description of one endopeptidase with specific properties obtained from one specific species of microorganism, but contains no specific description for the vast majority of the claim-encompassed endopeptidases from any possible source. More particularly, the Examiner alleges that the specification only describes one species, *S. pyogenes*, as the only microbial cells which applicant actually demonstrates producing the claimed endopeptidase having the claimed characteristics. The Examiner notes that the specification discloses that an extract of *S. aureus* showed similar activity; however, it is unclear if this enzyme is the same as the endopeptidase isolated from *S. pyogenes*. Thus, the Examiner alleges that the claims encompass numerous endopeptideases that can be produced by potentially any organism for which no specific written description has been provided. The Examiner asserts that given the huge variation in properties of endopeptidases and organisms producing said endopeptidases that are encompassed by the current broad claim language, the sole example of one endopeptidase having the claimed characteristics produced by *S. pyogenes* does not provide a representative sample of the endopeptidases and organisms producing said endopeptidases encompassed by the claims.

Applicants respectfully traverse the Examiner's rejection and have amended claim 1 to recite that the glycosylated enzyme is isolated from *Streptococcus* and *Staphylococcus* and that the LPXTG motif is found in cell surface proteins of these bacteria, whereby these cell surface proteins are virulence factors. A peptide having this motif was prepared in the present application (see page 5, paragraph [0019] and SEQ ID NO: 2) from the M protein of *Streptococcus* and was iodinated so that the cleavage of this peptide and the generation of particular fragments could be monitored after exposure of the peptide to the enzyme. It was noted that cleavage of free protein substrate (protein not bound to beads that have been coated with the protein substrate), such as that provided in SEQ ID NO: 2, occurred after the serine at position 6 of SEQ ID NO: 2 and after the glutamic acid at position 9 of SEQ ID NO: 2.

Moreover, Applicants submit that an enzyme with similar activity was identified in *Staphylococcus*, as noted in the present application on page 10, paragraph [0042]. While the data was not submitted with the present application, Applicants herewith submit a Declaration under 37 CFR §1.132, signed by Dr. Vincent Fischetti that attests to the fact that the enzyme was isolated from *Staphylococcus* and the activity and characterization of such enzyme was known at the time of filing of the present application. In addition, Dr. Fischetti has also used the same isolation protocol and has isolated and identified an enzyme from *Streptococcus pneumoniae* that has the ability to cleave at the same LPXTG motif as the *S. pyogenes* and *S. aureus* enzyme, and has attested to this in the attached Declaration under 37 CFR §1.132. Furthermore, the claims have been amended to recite that the enzyme is further characterized as having specific cleavage sites within a cell surface protein of such bacteria, in particular, a virulence factor, and more particularly, cleaving after the serine at position 6 of SEQ ID NO: 2 and after the glutamic acid at position 9 of SEQ ID NO: 2.

With respect to the rejections relating to claims 1-11, the mere fact that Applicants' specification does not teach every gram positive bacterium that possesses this enzyme, Applicants submit that given the showing that this enzyme is present in two different genera of gram positive bacteria, in particular, *Streptococcus* and *Staphylococcus,* and given the fact that other surface proteins in most gram positive bacteria become anchored to cell wall peptidoglycan by a common mechanism, it would be highly likely that this enzyme exists in most other gram positive bacteria (See reference by Navarre and Schneewind, Microbiology and Molecular Biology Reviews, March 1999, p. 174-229, attached herewith as EXHIBIT AA).

Further support for the presence of this enzyme in other bacteria is shown by the fact that an inhibitor of the LPXTGase enzyme was isolated and tested for inhibition of both LPXTGase enzyme activity and for its effect on inhibition of bacterial growth in the present application (see paragraphs [0117] – [0130] on pages 36-40). The results show that the inhibitor was effective in preventing the growth of a variety of gram positive bacteria, including various species of *Streptococcus*, *Staphylococcus* and *Bacillus*, as well as a gram negative bacterium, *E. coli*. This data supports the fact that bacteria other than *S. pyogenes* and *S. aureus* contain the LPXTGase enzyme, since an inhibitor of such enzyme was able to inhibit growth/virulence of a variety of organisms.

An enzyme called sortase has also been identified that cleaves the LPXTG motif, however, it differs from the present LPXTGase enzyme in that it cleaves in a different position (See Marraffini *et al*, Microbiology and Molecular Biology Reviews, March 2006, p. 192-221, attached herewith as EXHIBIT BB). The sortase enzyme cleaves the LPXTG motif after the threonine. Sortase has now been identified in nearly all gram-positive bacteria (See Marraffini *et al.*, EXHIBIT BB). The catalytic activity of the LPXTGase described in the present invention is 100x greater than sortase, suggesting that LPXTGase may be more active and important in the anchoring process.

However, in order to place the application in condition for allowance, Applicants have amended claim 1 to recite only *Streptococcus* and *Staphylococcus*. The guidelines for meeting the Written Description requirement clearly state that one must take into consideration all of the disclosed distinguishing identifying characteristics, including partial structure, physical and/or chemical characteristics, functional characteristics, known or disclosed correlation between structure and function, method of making, or any combinations of the above. One must weigh all of the factors in view of the level of one skilled in the art and in light of the written description. Under current law, the standard for meeting the Written Description requirement differs for every patent specification depending upon a number of factors, including the scientific knowledge in existence at the time of the invention, the skill in the art, the predictability of the claimed subject matter, and correlation of a described function to a known structure. *Capon v. Eshhar*, 418 F.3d 1349, 1357 (Fed. Cir. 2005).

Applicants submit that they have fully met this standard. Specifically, the maturity of the science and skill in the art at the time of the present invention were such that one of ordinary skill could predictably isolate and characterize the functional aspects of the enzyme from any gram positive bacterium, given the teachings in the present application. Moreover, as Applicants have pointed out in the specification on pages 30-31, in paragraphs [0101] to [0103], attempts were made to sequence the enzyme, using techniques that were known to those skilled in the art at the time of the present application. Applicants further assert that given the unique characteristics of this enzyme, and its unusual amino acid composition (as shown by acid hydrolysis of the isolated protein), precise sequencing using standard methodologies was impossible, thus, the best characterization of this enzyme could only be made based on its physical and/or biochemical characteristics, as well as its functional characteristics and protein cleavage pattern. Applicants further assert that they have provided the precise methods for isolating this enzyme and testing for its functional, as well as physical and biochemical characteristics, thus providing one skilled in the art with the teachings necessary to isolate and identify similar enzymes from other gram positive organisms.

Indeed, at the filing date of the present application (*i.e.*, in 2002), technologies for isolating and characterizing such proteins were highly developed, as were technologies for generating antibodies against such proteins. Once armed with an isolated protein, it was also well within the skill of the art to use these techniques to generate antibodies against such proteins and to utilize these antibodies for testing their cross-reactivity with proteins from different genera of gram positive bacteria to determine whether an enzyme having similar activity was the same as that isolated from another genus of gram positive bacteria.

Applicants specifically illustrated this by the procedures outlined in the present application and in the declaration and supporting documents submitted herewith. In particular, Applicants successfully isolated and characterized the enzyme from two different genera of gram positive bacteria, both of them known to possess virulence factors that aid in sustaining a successful infection in the host. In addition, Applicants successfully raised antibodies against the *Streptococcus* enzyme, which was then tested against the enzyme isolated from *Staphylococcus*, and the antibody reacted with both enzymes. This data is also included in the declaration executed by Dr. Fischetti. Applicants assert that the fact that an enzyme having similar activity was isolated from both *Streptococcus* and *Staphylococcus* bacteria, as indicated in the present application, coupled with the knowledge that an antibody to the enzyme isolated from *Streptococcus* cross-reacted with an enzyme having similar activity from *Staphylococcus* is certainly proof that the enzymes from *Streptococcus* and *Staphylococcus* are the same enzyme. This clearly indicates that Applicants had possession of the claimed invention. Moreover, the LPXTGase inhibitor described above and on pages 36-40 of the present application, clearly suppresses growth of not only *S. pyogenes*, but a variety of other gram positive and gram negative bacteria, suggesting that this enzyme is present in not only streptococcus and staphylococcus, but other organisms as well.

Applicants also describe well-known techniques for isolating and characterizing this enzyme. Based on these teachings, Applicants assert that it was well within the skill of the art to isolate and characterize the functional aspects of other enzymes having the same cleavage sites at the same motifs in other virulence factors from other gram positive or gram negative bacteria.

In fact, as evidenced by the original teachings in the present application, and by the additional supportive data in the Declaration signed by Dr. Vince Fischetti, the techniques for isolating the enzyme and characterizing its functional activity and cleavage sites in a virulence factor and further supportive data that shows that an antibody generated against the *Streptococcus* enzyme cross-reacted with the enzyme isolated by the same methods from *Staphylococcus*, provides ***clear evidence*** that Applicants were in fact indeed ***in possession of the claimed invention*** based on the descriptive text provided within the four corners of Applicants' originally filed disclosure.

In sum, the teachings set forth in Applicant's specification, in combination with the high level of skill and knowledge in the art at the time of the invention, and the proven predictability of the technologies involved in the invention, clearly satisfies the standard for Written Description according to the present guidelines for Written Description requirements and demonstrates possession of the claimed invention.

The Examiner has also rejected claims 1-11 under 35 U.S.C. §112, first paragraph for lack of enablement for the same reasons as noted above. In particular, the Examiner alleges that while the specification is enabled for an endopeptidase having the claimed MW, pH optima, motif cleavage specificity and glycosylation characteristics recited in claims 1, 2 and 7-11, wherein said endopeptidase is produced by *S. pyogenes*, the specification does not reasonably provide enablement for any and all endopeptidases having the claimed MW and pH optimum of claim 1 produced by any possible organism.

The Examiner asserts that the showing of one glycosylated endopeptidase with specific MW, pH optima and peptide-motif-cleavage specificity wherein the endopeptidases is produced by *S. pyogenes*, is not sufficient to enable a claim drawn to all endopeptidases characterized as having an apparent MW of 14 kD and a pH optima of 7.5 to 10 that is produced by any possible organism.

The Examiner alleges that there is no reliable method that predicts which organisms will produce the claimed endopeptidase or how to find an endopeptidase having a MW and pH optima recited in claim 1 that has the cleavage activity of a specific peptide-motif as described in the specification. The Examiner alleges that it would take undue experimentation to identify all of the organisms based on the broad scope of the claim, as currently pending. The Examiner further alleges that the existence of the claimed enzyme in other gram positive bacteria is speculative at best.

Applicants respectfully traverse the Examiner's rejection, but in order to place the application in condition for allowance, have amended the claims to recite only the two genera of gram positive bacteria demonstrated to have an LPXTGase enzyme with the activity and characteristics identified by the teachings of the present application. More particularly, the claims have been amended to recite only *Streptococcus* and *Staphylococcus*. However, Applicants assert that given the presence of the LPXTG motif in the surface proteins of nearly all gram-positive bacteria examined (See Table 1 on pages 185-186 of EXHIBIT AA, Navarre and Schneewind, Microbiology and Molecular Biology Reviews, March 1999), and that this motif is critical for surface protein expression, it is likely that the particular species within each of the genera claimed will have this enzyme. For example, while the present application teaches as particular examples, the enzyme from *Streptococcus pyogenes* and *Staphylococcus aureus*, further work done in the inventor's laboratory have also demonstrated the presence of the LPXTGase enzyme in *Streptococcus pneumoniae*. This work is attested to in the attached declaration under 37 CFR 1.132, signed by Dr. Fischetti in support of the currently amended claims. Furthermore, Applicants assert that this is the first enzyme ever reported that contains D-amino acids (specifically D-alanine) and contains unconventional amino acids. Thus, it is easy by those familiar with the art, to determine if the enzyme they have isolated cleaves the LPXTG motif as the inventors have described, and show that the enzyme contains D-amino acids (specifically D-alanine) and unconventional amino acids. This is not an ordinary endopeptidase but one that is unique based on these features.

For the reasons noted above, and in light of the current amendments to the claims, withdrawal of the rejections under 35 U.S.C. §112, first paragraph, is respectfully requested.

Rejection under 35 U.S.C. §112, second paragraph

Regarding the phrase "...characterized at cleaving at a ...motif"

Claims 2 and 7-11 were rejected under 35 U.S.C. 112, second paragraph, as being indefinite. In particular, the Examiner alleges that claim 2 is rejected because the phrase "...characterized at cleaving at a ...motif" is vague and indefinite. It is unclear what the motif is part of.

Applicants respectfully traverse the Examiner's rejection and have amended the claims to better clarify the invention. In particular, claim 1 has been amended to include the language of claim 2, with particular modifications to better clarify the cleavage sites within proteins having the LPXTG motif and to better define that the "motif" is present in a cell surface virulence factor from the bacterium.

Accordingly, claim 1 now recites:

> *"An isolated, glycosylated endopeptidase enzyme, wherein said enzyme is isolated from a Streptococcus or a Staphylococcus bacterium, and is characterized as having a pH optimum between 7.5 and 10, an apparent molecular weight of 14,000 Da, and is further characterized as cleaving at a site within, or C terminal to, a Leu Pro Xaa Thr Glu (LPXTG) (SEQ ID NO: 1) motif present in a cell surface protein of said bacterium, wherein said cell surface protein is a virulence factor of said bacterium, and wherein the cleaving results in enhanced virulence of the bacterium."*

Furthermore, a peptide was prepared from a virulence factor from S*treptococcus* (the M protein), and this peptide, designated as SEQ ID NO: 2, contains this LPXTG motif. The particular cleavage sites within the virulence factor are also further defined in amended claim 2, whereby it recites:

"The enzyme of claim 1, further characterized as cleaving after the serine at position 6 of SEQ ID NO: 2 and after the glutamic acid at position 9 of SEQ ID NO: 2."

Regarding the phrase "as lacking aromatic amino acids"

Claim 7 is rejected because the Examiner alleges that the phrase "as lacking aromatic amino acids" is vague and indefinite. The Examiner alleges that it is unclear if the phrase means that the enzyme does not have any aromatic amino acids or if the enzyme lacks aromatic amino acids compared to another enzyme.

Applicants respectfully traverse the Examiner's rejection and have amended claim 7 to better clarify that Applicants' intention was to characterize the enzyme as having no aromatic amino acids. Support for this amendment can be found on page 3, in paragraph [0009]; and further on page 26, paragraph [0089]. Accordingly, claim 7, as amended, recites the following:

"The enzyme of claim 5, wherein the enzyme is further characterized as having no aromatic amino acids, as having no cysteine or methionine, as not being inhibited by sulfhydryl agents, as being inhibited by hydroxylamine, as having a backbone structure

containing about 30% unknown amino acids and as having a K_m *of 0.26mM."*

Regarding the phrase "rich in alanine"

The Examiner also alleges that the phrase "rich in alanine" is vague and indefinite because "rich" is a relative term and no comparison is given. Applicants respectfully traverse the Examiner's rejection and have deleted this phrase from the claim, as originally filed. Applicants have further amended the claim to include the phrases:

"... as having no cysteine or methionine,..."

Support for this amendment can be found in the specification on page 10, paragraph [0042].

Regarding the phrase "salt sensitive"

The Examiner also alleges that the phrase "salt sensitive" is vague and indefinite because the metes and bounds of the sensitivity are unclear. Applicants respectfully traverse the Examiner's rejection and have amended claim 7 as noted above, which now recites:

"... as being inhibited by hydroxylamine at a concentration of about 100 mM..."

Support for this amendment can be found in Table 1 on page 41. Further support for this can be found in the specification in paragraph [0042] on page 10.

Regarding the Molar Ratios of the Carbohydrates

The Examiner has rejected claim 11 because it is not clear if the molar ratio expressed in claim 11 is based on the order of the carbohydrates named in claim 10.

Applicants respectfully traverse the rejection and assert that the ratios noted in claim 11 are based on the order of the carbohydrates in claim 10, and as such, have amended claim 11 to better clarify that the molar ratios of the carbohydrates of claim 10 are respective to each other. Accordingly, claim 11 now recites:

"The enzyme of claim 10, wherein the carbohydrates are present in a molar ratio of 1:2:3:13:2:2, respectively."

Based on the foregoing amendments to the claims, withdrawal of the rejection based on 35 U.S.C. §112, second paragraph, as being indefinite, is respectfully requested.

Rejection under 35 U.S.C. §101

The Examiner has rejected claims 1-11 under 35 U.S.C. §101, and alleges that the claimed invention is directed to non-statutory subject matter.

Applicants respectfully traverse the examiner's rejection and have amended the independent claim from which all other claims depend to recite the word "isolated". As such, Applicants assert that the amendment obviates the Examiner's rejection under 35 U.S.C. §101. Accordingly, withdrawal of the rejection is respectfully requested.

Rejection under 35 U.S.C. §102

The Examiner has rejected claims 1 and 3-6 under 35 U.S.C. §102(b) as being anticipated by Watanabe, *et al.* (1981).

The Examiner's Position Regarding Watanabe et al.

In particular, the Examiner alleges that Watanabe, et al., disclose an endopeptidease having a pH optima of 8.5 (maximal lytic activity) and a molecular weight estimated to be about 16,000 Daltons (abstract). The Examiner alleges that this disclosure meets the limitations of claim 1 because an endopeptidase having a pH optima that fall between 7.5 and 10 is disclosed. Furthermore, the Examiner alleges that the claimed MW is about 14,000 Daltons and since the specification does not define the term "about", the estimated MW of the endopeptidase disclosed by Watanabe, *et al.*, is about 14,000. Moreover, the Examiner alleges that while claims 3-6 are drawn to the source of the enzyme, these claims do not recite any actual properties that differentiate an endopeptidase isolated from any of the sources named in claims 3-5 from the endopeptidase taught by Watanabe, *et al.*

Applicants' Invention as Currently Claimed

Applicants respectfully traverse the Examiner's rejection and have further amended claim 1 to note that the enzyme is isolated from *Streptococcus* and *Staphylococcus* and is glycosylated. Furthermore, the term "about" has been deleted. The claim has also been amended to include the language from claim 2, and more particularly notes that the enzyme is further characterized as cleaving at a site within, or C terminal to, a Leu Pro Xaa Thr Glu (LPXTG) (SEQ ID NO: 1) motif present in a cell surface protein of said bacterium. Furthermore, claim 2 has also been amended to note additional aspects as related to the specific cleavage sites within the virulence factor of the bacterium.

More particularly, as noted previously, claim 1 now recites:

> *"An isolated, glycosylated endopeptidase enzyme, wherein said enzyme is isolated from a Streptococcus or a Staphylococcus bacterium, and is characterized as having a pH optimum between 7.5 and 10, an apparent molecular weight of 14,000 Da, and is further characterized as cleaving at a site within, or C terminal to, a Leu Pro Xaa Thr Glu (LPXTG) (SEQ ID NO: 1) motif present in a cell surface protein of said bacterium, wherein said cell surface protein is a virulence factor of said bacterium, and wherein the cleaving results in enhanced virulence of the bacterium."*

Applicants' Position Regarding Watanabe *et al.*

Applicants assert that Watanabe *et al.* do not teach or suggest the enzyme of the present application for the following reasons.

The enzyme taught by Watanabe et al. is a **lytic** enzyme isolated from *Acromobacter lunatus*, which demonstrates lytic activity on *Micrococcus radiodurans* and *Pseudomonas radiora*. **The enzyme of the present application is not a lytic enzyme that cleaves at the sites indicated by Watanabe *et al.* in Figure 8 of the reference cited.**

Applicants assert that Watanabe et al. **do not teach or suggest** that their enzyme is an isolated, **glycosylated** endopeptidase enzyme **isolated from a *Streptococcus* or a *Staphylococcus* bacterium and characterized as cleaving at a site within, or C terminal to, a Leu Pro Xaa Thr Glu (LPXTG) (SEQ ID NO: 1) motif present in a cell surface protein of said bacterium, wherein the cell surface protein is a virulence factor of said bacterium, and wherein the cleaving results in enhanced virulence of the bacterium.**

In addition, Watanabe *et al* **do not teach or suggest that their enzyme has a molecular weight of 14,000 daltons or that it cleaves after the serine at position 6 of SEQ ID NO: 2 and after the glutamic acid at position 9 of SEQ ID NO: 2.**

Applicants have also canceled claims 3 and 4, thereby rendering the rejection moot based on these claims. However, based on the current amendments to the claims, Applicants assert that Watanabe *et al.* do not teach or suggest the enzyme of the present invention for the reasons noted above. Withdrawal of the rejection is respectfully requested.

Fees

A check in the amount of $225.00 is enclosed to cover two new independent claims and one new dependent claim as a small entity. No other fees are believed to be necessitated by the foregoing amendment and response. However, if this is in error, authorization is hereby given to charge Deposit Account No. 11-1153 for any underpayment, or credit any overages.

Conclusion

Applicants believe that the foregoing amendments to the claims and arguments place the application in condition for allowance. Withdrawal of the rejections is respectfully requested. If a discussion with the undersigned will be of assistance in resolving any remaining issues, the Examiner is invited to telephone the undersigned at (201) 487-5800, ext. 118, to effect a resolution.

Respectfully submitted,

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Attachments: EXHIBIT AA (Navarre and Schneewind, Microbiology and Molecular Biology reviews, March 1999, p. 174-229;

EXHIBIT BB (Marraffini et al., Microbiology and Molecular Biology reviews, March 2006, p. 192-221)

DECLARATION under 37 CFR §1.132 with EXHIBIT A (CV of Dr. Fischetti), EXHIBIT B (paper by Lee et al. J. Biol. Chem. (2002), Vol. 277, No. 49, pp. 46912-46922), EXHIBIT C (Materials, Methods and Results related to isolation and characterization of the Staphylococcal enzyme)

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Sortases and the Art of Anchoring Proteins to the Envelopes of Gram-Positive Bacteria

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INTRODUCTION

The cell wall envelopes of gram-positive bacteria represent a surface organelle that not only functions as a cytoskeletal element for the physical integrity of microbes but also promotes interactions between bacteria and their environment (60). Most importantly for bacterial pathogens, as environments are subject to change, microbes respond with alterations in envelope structure and function. Thus, one should consider the cell wall envelope a dynamic organelle, one that is continuously assembled from precursor molecules and disassembled into individual constituents.

Bacterial cell wall assembly requires peptidoglycan precursors that together form a single large macromolecule, the murein sacculus, encircling the microbial cell with a 20- to 100-nm-thick wall structure (61). Cell wall peptidoglycan is covalently and noncovalently decorated with teichoic acids, polysaccharides, and proteins. The sum of these molecular decorations provide bacterial envelopes with species- and strain-specific properties that, for pathogens, contribute greatly to bacterial virulence, interactions with host immune systems, and the development of disease symptoms or successful outcomes of infections. This review focuses on the mechanisms of surface protein anchoring to the cell wall envelope by sortases and the roles that these enzymes play in bacterial physiology and pathogenesis. Interested readers are referred to other excellent reviews that have examined in depth the structure and assembly of peptidoglycan, teichoic acids, and polysaccharides or proteins that are noncovalently associated with the cell wall envelope (136, 139, 144, 187).

In *Staphylococcus aureus*, peptidoglycan precursor molecules are fabricated from *N*-acetylmuramic acid (MurNAc) and L- as

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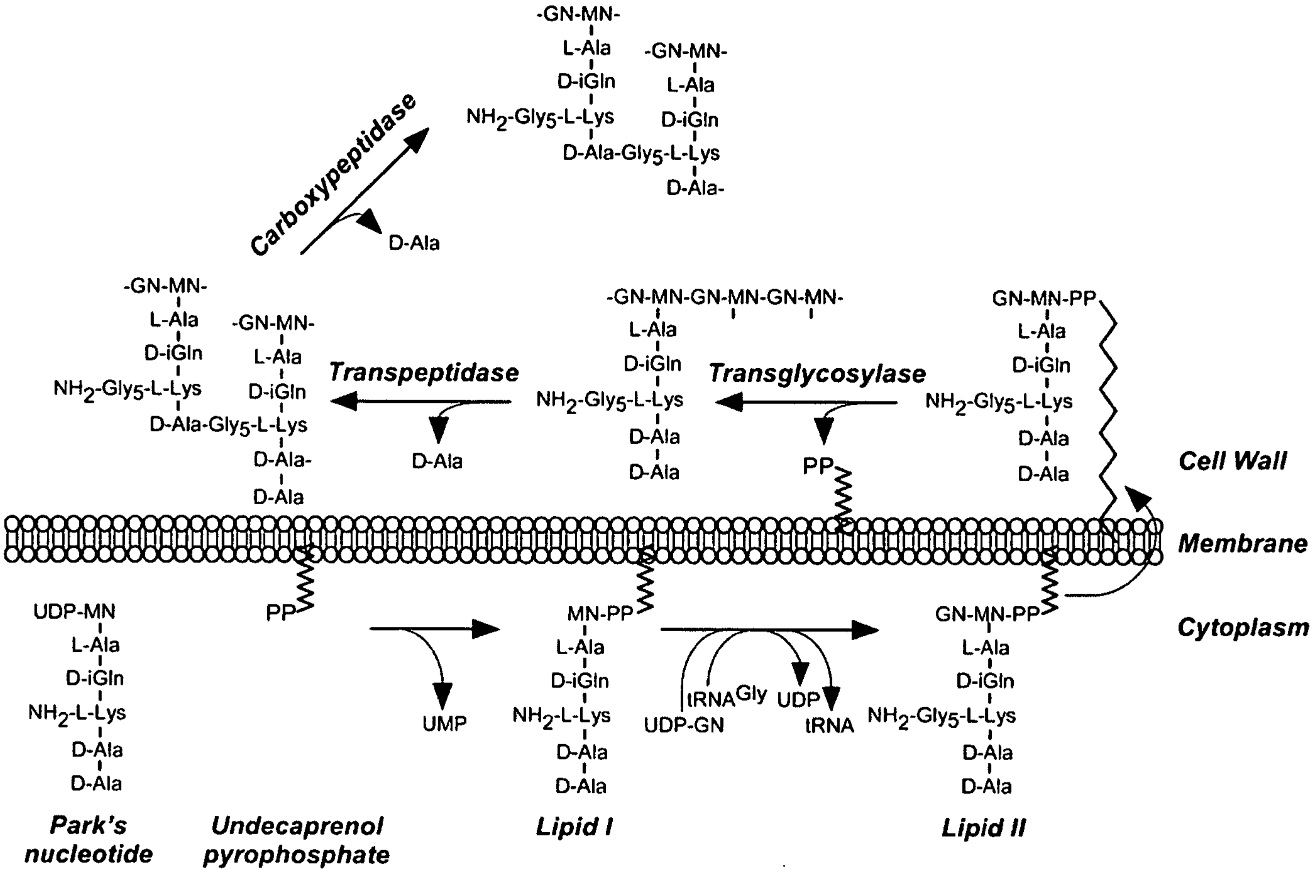

FIG. 1. Peptidoglycan synthesis in *S. aureus*. Park's nucleotide, a soluble nucleotide precursor, originates in the bacterial cytoplasm by successive addition of L-stereoisomer amino acids (L-Ala and L-Lys) as well as D-stereoisomer amino acids (D-isoglutamine [D-iGln] and D-Ala) to UDP-*N*-acetylmuramic acid (UDP-NM). Precursor transfer to undecaprenol pyrophosphate, a bacterial membrane carrier, generates lipid I and removes UMP nucleotide. Lipid I modification with *N*-acetylglucosamine (GN) and pentaglycine cross bridge formation at the ε-amino of L-Lys with tRNA^{Gly} substrate generates lipid II. Following translocation across the cytoplasmic membrane, lipid II serves as substrate for PBPs that catalyze three reactions: transglycosylation, transpeptidation, and carboxypeptidation. Transglycosylases polymerize MN-GN subunits into repeating disaccharide chains, the glycan strands. Transpeptidases cleave the amide bond of the terminal D-Ala in pentapeptide precursors and generate an amide bond between the carboxyl group of D-Ala at position four and the amino group of pentaglycine cross bridges in wall peptides. Carboxypeptidases hydrolyze the C-terminal D-Ala of most non-cross-linked pentapeptides to yield mature peptidoglycan.

well as D-stereoisomer amino acids in the bacterial cytoplasm to yield a soluble intermediate, Park's nucleotide (UDP–MurNAc–L-Ala–D-isoGln–L-Lys–D-Ala–D-Ala) (24) (Fig. 1). The precursor is tethered via phosphodiester linkage to a bactoprenol carrier, generating lipid I (C_{55}-PP–MurNAc–L-Ala–D-isoGln–L-Lys–D-Ala–D-Ala) in the membrane (24, 117, 118). Further modification with *N*-acetylglucosamine (GlcNAc) and cross bridge decoration at the ε-amino of L-Lys (pentaglycine or Gly_5 in staphylococci) generates lipid II {C_{55}-PP–MurNAc–[L-Ala–D-isoGln–L-Lys(Gly_5)–D-Ala–D-Ala]–β(1-4)-GlcNAc)}. Lipid II is translocated across the cell membrane (133), where it becomes a substrate for penicillin binding proteins (PBPs) that catalyze transglycosylation and transpeptidation reactions. Transglycosylation polymerizes MurNAc-GlcNAc subunits into repeating disaccharide chains, also called glycan strands (194). Transpeptidation involves first cleavage of the pentapeptide precursor [L-Ala–D-isoGln–L-Lys(Gly_5)–D-Ala–D-Ala] at the terminal D-Ala and then formation of an amide bond between the carboxyl group of D-Ala at position four and the amino groups of pentaglycine cross bridges in other wall peptides (85). PBPs use these two reactions together to form a single large macromolecule that displays rigid exoskeletal functions and that serves as a scaffold for the incorporation of other molecules that can be attached to cross bridges, wall peptides, or glycan strands. Peptidoglycan biosynthesis in other bacteria follows a similar scheme, with two exceptions. First, D-isoGlu at position two of wall peptides is typically not amidated. Second, L-Lys, the diamino acid at position three of wall peptides, can be substituted with *m*-diaminopimelic acid, and the attached cell wall cross bridges can vary in chemical nature between different bacterial species (170).

Sortases promote the covalent anchoring of surface proteins to the cell wall envelope (120). These enzymes catalyze a transpeptidation reaction by first cleaving a surface protein substrate at the cell wall sorting signal. The resulting acyl enzyme intermediates between sortases and their substrates are then resolved by the nucleophilic attack of amino groups, typically provided by the cell wall cross bridges of peptidoglycan precursors. The product of the sortase reaction, a surface protein linked to peptidoglycan, is then incorporated into the envelope and displayed on the microbial surface. Surface proteins typically carry two topogenic sequences, N-terminal signal peptides and C-terminal sorting signals. Cell wall sorting signals span approximately 30 to 40 residues and comprise a short pentapeptide motif followed by a stretch of hydrophobic side chains and finally a mostly positively charged tail at the C-terminal end of the polypeptide (174). Sortase is a central factor in the so-called "sorting pathway." This pathway begins with the synthesis of a surface protein precursor in the cytoplasm. The N-terminal signal peptide then directs the precursor to the membrane for translocation (1). Once the signal peptide has been cleaved and the polypeptide is moved across the plasma membrane, the cell wall sorting signal functions to retain the polypeptide within the secretory pathway. Membrane-anchored sortases cleave sorting signals at their pentapeptide motif and promote anchoring to the cell wall (120).

Recent discoveries have shown that sortases catalyze diverse transpeptidation reactions using specific polypeptide or peptidoglycan substrates. Further, sortases can target unique domains of the bacterial cell wall envelope and can even promote the assembly of pili in gram-positive bacteria. These discoveries are discussed here in the context of current research frontiers. The underlying contributions of surface proteins and sortases to the pathogenesis of bacterial infections have been revealed in animal models of disease, and these findings may be exploited for the implementation of new therapeutic strategies.

SURFACE PROTEINS, THE SUBSTRATES OF SORTASE

Staphylococcus aureus Surface Proteins and Their Functions

Staphylococcus aureus is a human and animal pathogen that causes diverse infections. As a resident of the human skin, nails, and nares, this microbe has the unique ability to penetrate deeper layers of host barriers, generating suppurative lesions in virtually all organ systems. Staphylococci lack pili or fimbrial structures and instead rely on surface protein-mediated adhesion to host cells or invasion of tissues as a strategy for escape from immune defenses (53). Furthermore, *S. aureus* utilizes surface proteins to sequester iron from the host during infection (182). The majority of surface proteins involved in these aspects of staphylococcal disease are sortase substrates; i.e., they are covalently linked to the cell wall by sortase (Fig. 2).

Sequence comparison of cloned surface proteins of gram-positive bacteria provided the first insight for the existence of a signal involved in anchoring these polypeptides within the envelope (51). These studies first identified six surface proteins with a common motif sequence, now referred to as LPXTG motif-type sorting signals. The sequencing of microbial genomes has greatly expanded our knowledge of the repertoire of surface proteins. Recent analyses of available sequences indicated that 732 surface protein genes carry C-terminal cell wall sorting signals in 49 microbial genome sequences (12). Here we provide a brief synopsis of what is known about surface proteins of *S. aureus*, molecules that have been studied for more than 50 years.

Using cell wall sorting signals as queries in bioinformatic searches, 18 to 22 genes encoding putative sortase-anchored surface proteins were identified in the genomes of *S. aureus*, varying with the strain under investigation (see Table 1 for a listing of 22 surface proteins) (62, 122, 123, 162). Microbial surface components recognizing adhesive matrix molecules (MSCRAMMs) are bacterial elements of tissue adhesion and immune evasion (53). The study of several staphylococcal proteins has helped lay the foundation of our current understanding of these molecules, and these include the fibronectin binding proteins FnbpA and FnbpB (52, 89, 166, 178). Both proteins encompass a large N-terminal domain (about 500 amino acid residues) followed by four or five 50-residue repeat domains responsible for binding the N-terminal domain of fibronectin. FnbpA/FnbpB interactions with fibronectin involve structural rearrangements that lead to the ordering of the Fnbp repeat domains upon ligand binding (89, 162, 212). As fibronectin is found in extracellular matrices of most tissues as well as in soluble form within body fluids, staphylococci can adhere to virtually all tissues or serum-coated foreign bodies (151). With such widespread binding potential, one important aspect of staphylococcal binding to fibronectin is the invasion of host cells and subsequent intracellular replication (8, 44).

Staphylococcal strains causing connective tissue infections or osteomyelitis regularly express the collagen adhesion protein (Cna) (152, 190). A large N-terminal domain encompasses the binding site for collagen, the A domain, which assembles with a jellyroll fold (161). A molecular trench within this fold can accommodate the collagen triple helices.

S. aureus strains clump in the presence of plasma. This phenomenon, which has been exploited for diagnostic purposes, is the product of a molecular interaction between two MSCRAMMs, clumping factors A and B (ClfA and ClfB), and fibrinogen (54, 124, 141). ClfA and ClfB are structurally related and comprise a large N-terminal A domain and a repeat domain (R domain) which is composed exclusively of serine-aspartate repeats (69, 90). The ligand binding sites of ClfA and ClfB have been mapped to residues 220 to 559 (125), which assume an immunoglobulin G (IgG)-like fold (37, 125, 153, 209). An elegant molecular mechanism of fibrinogen substrate binding, coined "dock, lock, and latch," has recently been demonstrated for SdrG, a fibrinogen binding *Staphylococcus epidermidis* MSCRAMM that also encompasses repeat domains (156). A cleft of 30 Å in length between two IgG-like folds of SdrG constitutes the fibrinogen binding site, with at least 62 contacts between the two molecules that occlude the cleavage sites for thrombin. Both *S. aureus* and *S. epidermidis* strains encode multiple cell wall-anchored surface proteins with large serine-aspartate repeat (Sdr) domains (69, 90, 156). Other surface proteins containing Sdr domains include the aforementioned ClfA and ClfB but also SdrC, SdrD, and SdrE. The B domains of Sdr proteins contain high-affinity calcium binding sites which adopt an EF hand fold, a common structure observed in other calcium binding proteins (91, 205). Although it seems likely that these proteins are involved in binding host factors, such interactions have thus far not been demonstrated for the majority of the Sdr proteins.

S. aureus protein A (Spa) binds to the Fc termini of mammalian immunoglobulins in a nonimmune fashion, resulting in the uniform coating of staphylococci with antibodies (86). The

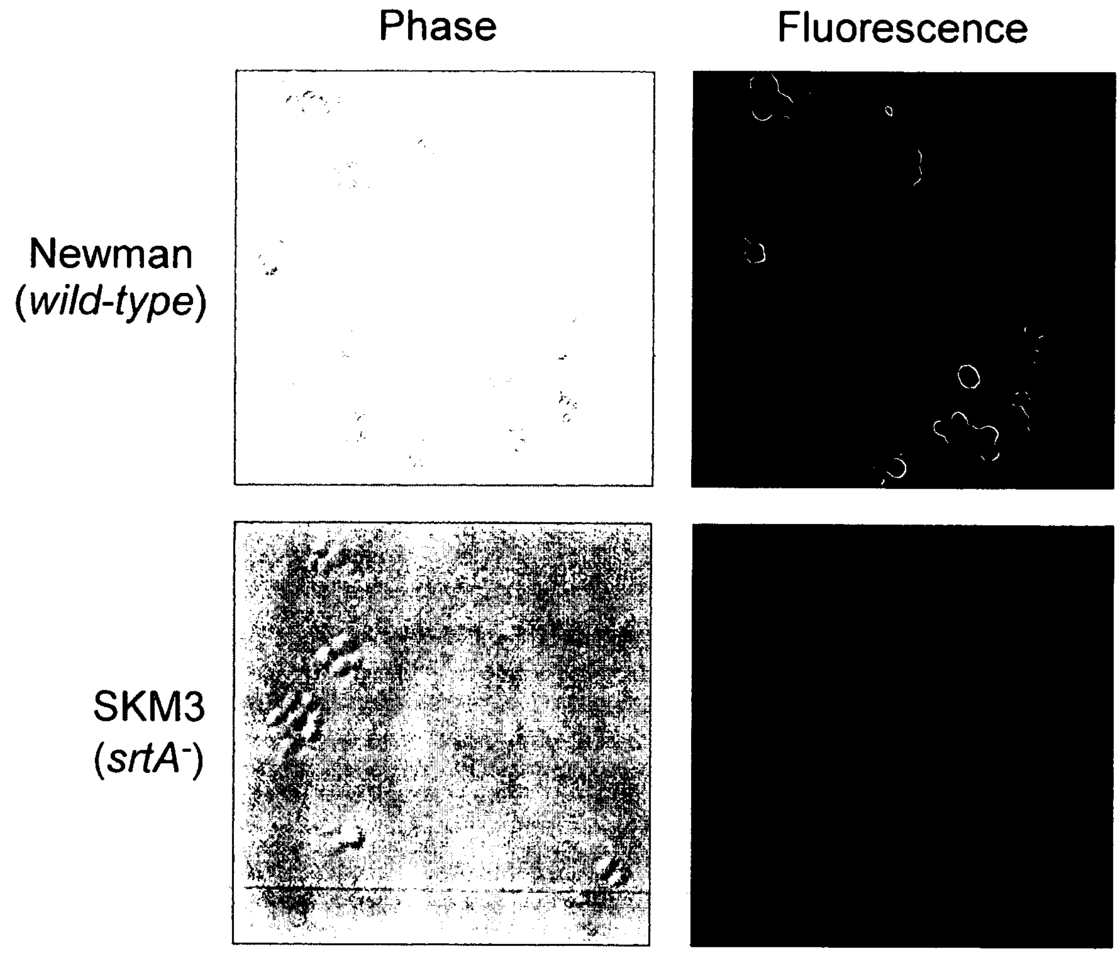

FIG. 2. Sortase A-dependent surface display of staphylococcal proteins. Sortase is responsible for the anchoring of 20 different surface proteins to the cell wall of *S. aureus* strain Newman. One of these surface proteins, protein A, binds to the Fc terminus of mammalian immunoglobulins in a nonimmune fashion, causing decoration of the staphylococcal surface with antibody. Using Cy3-conjugated immunoglobulin and *S. aureus* strain Newman, protein A display on the bacterial surface was revealed with phase-contrast microscopy and fluorescence microscopy. Protein A display on the staphylococcal surface is abrogated in the *srtA* mutant strain (SKM3).

protein A amino acid sequence, gene sequence, and three-dimensional nuclear magnetic resonance and X-ray diffraction structures revealed a molecule comprised of five nearly identical immunoglobulin binding domains (36, 65, 179, 206). Mutations in the protein A gene (*spa*) cause significant defects in the pathogenesis of *S. aureus* infections. For example, reduced bacterial survival in blood or in the presence of macrophages is likely due to the inability of these variants to sequester immunoglobulin via Fc binding (149). However, the observed phenotypes may also be attributed to defects in the binding of protein A to von Willebrand factor, a serum polypeptide that promotes physiological homeostasis of human or animal blood, or to protein A binding to tumor necrosis factor receptor 1, a signaling molecule involved in proinflammatory cytokine responses and innate immunity (64, 70).

Four Isd proteins (iron-regulated surface determinants) are involved in binding heme or hemoproteins and appear to play a role in iron scavenging during staphylococcal host infection. HarA/IsdH is encoded by a gene outside the *isd* locus (see below) and has been shown to bind haptoglobin/hemoglobin complexes (42). IsdB, on the other hand, binds to hemoglobin, and four proteins, i.e., IsdA, IsdB, IsdC, and IsdH/HarA, bind heme (121, 182). It has been proposed that these proteins are involved in capturing hemoproteins on the bacterial surface, liberating heme, and promoting heme transport across the bacterial cell wall envelope (182). The functions of twelve *S. aureus* surface proteins with C-terminal sorting signals, i.e., SasA, SasB, SasC, SasD, SasF, SasG, SasH, SasK, SdrC, SdrD, SdrE, and Pls, are not yet known. Table 1 summarizes the current knowledge about *S. aureus* surface proteins.

Signal Peptides and Cell Wall Sorting Signals

All cell wall-anchored surface proteins of staphylococci or other gram-positive bacteria encode at least two topogenic sequences, an N-terminal signal peptide and a C-terminal cell wall sorting signal. For example, the N-terminal signal peptide of protein A is necessary for the secretion of precursor proteins via the Sec pathway of staphylococci and is sufficient to promote the secretion of other signal peptide-less reporter pro-

TABLE 1. *Staphylococcus aureus* cell wall-anchored surface proteins

Surface protein	aa[a]	Ligand(s)[b]	Motif[c]	Sortase[d]	Reference(s)
Protein A (Spa)	508	Immunoglobulin, von Willebrand Factor, TNFR[e]	LPETG	A	64, 70, 206
Fibronectin binding protein A (FnbpA)	1,018	Fibronectin, fibrinogen, elastin	LPETG	A	178
Fibronectin binding protein B (FnbpB)	914	Fibronectin, fibrinogen, elastin	LPETG	A	89
Clumping factor A (ClfA)	933	Fibrinogen	LPDTG	A	124
Clumping factor B (ClfB)	913	Fibrinogen, keratin	LPETG	A	141
Collagen adhesion (Cna)	1,183	Collagen	LPKTG	A	152
SdrC	947	Unknown	LPETG	A	90
SdrD	1,315	Unknown	LPETG	A	90, 91
SdrE	1,166	Unknown	LPETG	A	90
Pls	1,637	Unknown	LPDTG	A	122, 123
SasA	2,261	Unknown	LPDTG	A	122, 123
SasB	937	Unknown	LPDTG	A	122, 123
SasC	2,186	Unknown	LPNTG	A	122, 123
SasD	241	Unknown	LPAAG	A	122, 123
SasE/IsdA	354	Heme	LPKTG	A	121–123
SasF	637	Unknown	LPKAG	A	122, 123
SasG/Aap	1,117	Unknown	LPKTG	A	78, 122, 123
SasH	308	Unknown	LPKTG	A	122, 123
SasI/HarA/IsdH	895	Haptoglobin	LPKTG	A	42, 121–123
SasJ/IsdB	645	Hemoglobin, heme	LPQTG	A	121–123
SasK	211	Unknown	LPKTG	A	122, 123
IsdC	227	Heme	NPQTN	B	110, 121

[a] aa, protein length in amino acids.

[b] Molecular component(s) recognized and bound by protein.

[c] Consensus motif recognized by sortase and present in C-terminal cell wall sorting signal.

[d] Sortase for which cell wall surface protein is substrate.

[e] TNFR, tumor necrosis factor receptor.

teins (1, 4). Signal peptidase cleaves the protein A signal peptide between residues 36 and 37 (174). Following translocation across the plasma membrane, the N-terminal portion of protein A is displayed on the bacterial surface, whereas the C-terminal end is buried in the cell wall peptidoglycan and protected from extracellular protease (67). The protein A signal peptide is a member of the YSIRK-G/S family of signal peptides, which can be found in some but not all surface proteins of gram-positive bacteria and in a few secreted polypeptides (164, 192). Removal of the YSIRK-G/S motif does not abrogate the cell wall anchoring and surface display of mutant protein A; however, the rate of surface protein anchoring to the cell wall envelope is somewhat diminished (4). Clearly, signal peptides of other surface proteins or secreted polypeptides and even type II signal peptides triggering diacyl-glycerol decoration do not interfere with the function of cell wall sorting signals (134).

The C-terminal cell wall sorting signal of staphylococcal protein A encompasses a 35-residue peptide with an LPXTG motif, followed by a hydrophobic domain and a positively charged tail (173). Mutations that truncate the sorting signal cause the secretion of mutant protein A into the extracellular medium. In contrast, mutations that delete or substitute residues within the LPXTG motif abolish sortase-mediated cell wall linkage without secretion of mutant protein A (174). The cell wall sorting signal alone is sufficient to cause cell wall anchoring of other polypeptides that are initiated into the secretory pathway of *S. aureus* via an N-terminal signal peptide (38, 134, 135, 195). Moreover, sorting signals from one species can be functional in another microorganism (173). When the sorting function fails, mutations that either alter the distance between the LPXTG motif and the charged tail or affect residues within the two parts of the sorting signal repair the lack of function of the heterologous cell wall sorting signal (173).

Cell wall sorting signals are functional even if they do not reside at the C-terminal end of the polypeptide chain (135). Nevertheless, sorting signal function absolutely requires an upstream signal peptide. Positioning the cell wall sorting signal in the middle of an engineered polypeptide, flanked at its N-terminal side by the signal peptide-bearing reporter staphylococcal enterotoxin B (Seb) and at its C-terminal border with the mature domain of β-lactamase (BlaZ), generates a hybrid precursor that is cleaved at the N-terminal signal peptide and initiated into the secretory pathway (135). The precursor is then cleaved between the threonine and the glycine of the LPXTG motif, and the N-terminal portion of the precursor is tethered to the cell wall envelope. In contrast, the C-terminal portion of the precursor with the remainder of the cleaved cell wall sorting signal resides in the bacterial cytoplasm.

Sorting signals have been observed in a plethora of predicted gene products, most of which were identified via genome sequencing of gram-positive bacteria (12, 31, 51, 122, 136, 148). While the great majority of these sorting signals carry the LPXTG motif, others harbor variations of this sequence (Table 2) (see below). If a surface protein gene that contains such variation resides in the same transcriptional unit with a sortase gene, it is generally presumed that the two genes encode an enzyme-substrate pair, i.e., that the sortase specifically recognizes and cleaves the sorting signal of the cotranscribed substrate. This conjecture has been experimentally confirmed for *Corynebacterium diphtheriae spa* loci (204), *S. aureus isd-srtB* (123), and *Listeria monocytogenes svpA-srtB* (10) (see below).

TABLE 2. Sortase classifications

Sortase class (subfamily)[a]	Cleavage site[b]	Membrane anchor domain[c]	Bacterial taxa[d]	References
A (1)	LPkT-Ge*	N terminus	*Bacillus, Listeria, Staphylococcus, Enterococcus, Lactobacillaceae, Streptococcaceae*	31, 41, 171, 197
B (2)	NPqt-nd*	N terminus	*Bacillus, Listeria, Staphylococcus, Streptococcaceae, Clostridia*	31, 41, 115
C (3)	IPkT-GG	C terminus	*Actinobacteria, Bacillus, Enterococcus, Leuconostocaceae, Streptococcaceae, Clostridia*	31, 41
D (4)	LPnT-At	N terminus	*Bacillus*	31, 41
D (5)	LAeT-Ga	N terminus	*Actinobacteria*	31, 41

[a] Sortase subfamily and class assignments are based on sequence, membrane topology, genomic positioning, and preference for specific amino acids within the cell wall sorting signal pentapeptide motif region of their cognate substrates (31, 41).

[b] Cell wall sorting signal pentapeptide motif. Uppercase letters represent amino acids that are absolutely conserved. Asterisks indicate that the cleavage site has been verified experimentally.

[c] Membrane anchor region based on transmembrane predictions and regions of high hydrophobicity.

[d] Bacterial taxa harboring one or more sortase genes belonging to the respective sortase clasification.

Anchor Structure of Staphylococcal Surface Proteins

Sjöquist and colleagues solubilized protein A from the bacterial envelope by treatment of peptidoglycan with lysostaphin, a glycyl-glycine endopeptidase that cleaves the pentaglycine of staphylococcal cell wall cross bridges (180). Initially a protein A domain known as region X was thought to promote binding to the cell wall envelope. This domain consists of a disordered structure composed of a variable number of 8-amino-acid repeats (67). However, region X alone cannot retain protein A or other polypeptides in the envelope, and deletion of this domain does not abolish protein A anchoring or surface display (173).

Muramidases cleave the glycan strands of staphylococcal peptidoglycan and release protein A as a spectrum of molecules with different masses. In contrast, lysostaphin releases protein A species with smaller masses (173). C-terminal anchor structures of protein A were deduced by analyzing engineered surface protein sortase substrates. The protein A cell wall sorting signal was fused to the C-terminal end of *Escherichia coli* maltose binding protein (MalE) (171). Cell wall-anchored MalE was released with lysostaphin from the staphylococcal envelope, purified, and cleaved with trypsin, and C-terminal peptides were analyzed by Edman degradation and mass spectrometry, which revealed the sequence LPET-Gly$_4$, LPET-Gly$_3$, and LPET-Gly$_2$ (171). As the cell wall sorting signal of protein A is cleaved between the threonine and glycine residues of the LPXTG motif, addition of glycine residues to the carboxyl-terminal end of protein A must be due to amide linkage of surface protein to the cell wall cross bridge of staphylococci, and this pentaglycine is cleaved by lysostaphin at positions 2, 3, and 4.

The complete anchor structure of surface proteins in staphylococci was determined after solubilization of peptidoglycan with muramidase, amidase, D-Ala–Gly endopeptidase, and lysostaphin (137, 138, 195). Seb-MHis$_6$-Cws, an engineered reporter comprised of Seb fused to the protein A cell wall sorting signal (Cws) via a methionyl-six-histidyl linker (MHis$_6$), can be solubilized from the peptidoglycan via cleavage with muralytic enzymes, purified by affinity chromatography on nickel-nitrilotriacetic acid resin, and then cleaved with cyanogen bromide at methionyl residues. C-terminal anchor peptides are purified by a second round of affinity chromatography and analyzed by mass spectrometry and Edman degradation. Using this technology, surface proteins were found to be linked to the cell wall cross bridges of cross-linked peptidoglycan units, comprised predominantly of murein tetrapeptides {MurNAc–[L-Ala–D-isoGln–L-Lys–(Gly$_5$)–D-Ala-]–GlcNAc}, and only rarely to murein-pentapeptides {MurNAc–[L-Ala–D-isoGln–L-Lys–(Gly$_5$)–D-Ala–D-Ala]–GlcNAc} that were released by muramidase cleavage of glycan strands or amidase cleavage of cell wall peptides. The overall picture that emerged from these studies indicates that surface proteins are embedded in peptidoglycan and occupy any position along glycan strands that are comprised of 2 to 11 disaccharide units and at any position along tetrapeptide cross-links with 1 to 15 wall peptide units (Fig. 3).

S. AUREUS SORTASE A

Molecular Genetic Analysis of Sortase A (*srtA*) Function

One thousand temperature-sensitive *S. aureus* mutants, generated by chemical mutagenesis, were transformed with a reporter plasmid providing for the expression of Seb-Spa$_{CWS}$, a

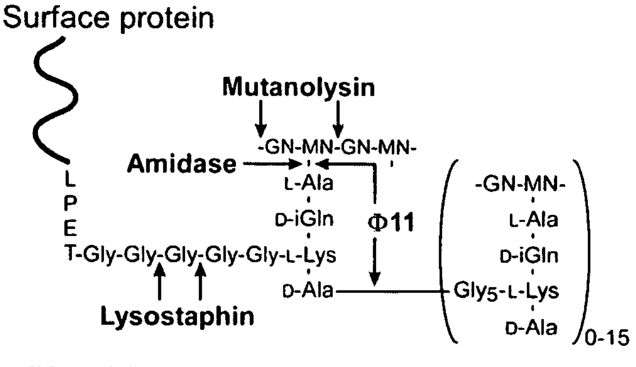

FIG. 3. Cell wall anchor structure of staphylococcal surface proteins. The C-terminal threonine of surface proteins, generated by sortase A-mediated cleavage between the threonine and the glycine of the LPXTG motif, is amide linked to the pentaglycine cross bridge of *S. aureus* cell wall peptidoglycan. Treatment of the staphylococcal peptidoglycan with lysostaphin (glycyl-glycine endopeptidase), mutanolysin [*N*-acetylmuramidase that cleaves the β(1-4) *O*-glycosidic bond between *N*-acetylmuramic acid and *N*-acetylglucosamine (GN)], amidase (*N*-acetylmuramoyl-L-Ala amidase), or Φ11 hydrolase (*N*-acetylmuramoyl-L-Ala amidase and D-Ala-Gly endopeptidase) releases surface protein with the predicted C-terminal cell wall anchor structures.

hybrid between enterotoxin B and the protein A cell wall sorting signal (120). Sortase-mediated cleavage of Seb-Spa_{CWS} was monitored by pulse-labeling experiments, and the anchoring efficiency of each mutant was scored. One variant, SKM317, with a significantly reduced rate of sorting signal cleavage was isolated. Using plasmid libraries for complementation studies in SKM317, the *srtA* gene was isolated and sequenced. Deletion of the *srtA* gene by homologous recombination results in a mutant that displays no defect in staphylococcal growth on agar or laboratory media. Pulse-labeling experiments showed that *srtA* mutants synthesize and secrete surface protein precursors but fail to cleave these polypeptides at their C-terminal cell wall sorting signals. As a consequence, *srtA* mutants do not display protein A, fibronectin binding proteins, or clumping factors on the bacterial surface, a phenotype that can be rescued by plasmid-encoded expression of the wild-type *srtA* gene (119). When analyzed with Seb reporter fusions to various sorting signals, *srtA* mutants are found to be defective in the cleavage of all staphylococcal sorting signals carrying LPXTG motif sequences (123).

Expression of several surface protein genes appears to be dramatically reduced in *S. aureus srtA* mutants, and the molecular mechanisms underlying this regulatory phenomenon have not yet been explored (S. K. Mazmanian and O. Schneewind, unpublished observation). Overexpression of plasmid-encoded surface protein genes or reporter genes encoding secreted proteins with C-terminal sorting signals greatly reduces the viability of staphylococci carrying *srtA* deletions (123). It seems plausible that *srtA* mutations cause the accumulation of surface proteins within the secretory pathway, which has recently been dubbed the ex-portal for *Streptococcus pyogenes* (163), a pathogen that is closely related to staphylococci. As these polypeptides cannot be cleaved in the absence of sortase and therefore cannot advance along the sorting pathway, it seems likely that they may block the ex-portal.

The contribution of *S. aureus srtA* to the pathogenesis of staphylococcal disease was examined in several different animal model systems of infection. *S. aureus* strain Newman, a human clinical isolate, was used as a parent, and the *srtA* gene was replaced with the erythromycin resistance cassette (119). Compared to the wild-type parent, sortase mutants displayed a 1.5-log-unit increase in the 50% lethal dose (LD_{50}) measured after intraperitoneal injection of staphylococci into mice, indicating a reduction in the virulence of the *srtA* strain. This defect may not seem large, especially compared to virulence genes in microbes that are particularly prone to causing lethal infections in mice, such as *Yersinia pestis* (155). However, the LD_{50} for *S. aureus* strain Newman is already high, requires about 10^7 CFU (119). Any reduction in virulence of staphylococci beyond 1 to 2 log units is concealed by an experimental ceiling with a lethal dose of about 10^8 to 10^9 CFU for any bacterial organism (dead or alive), because massive induction of innate inflammatory responses by bacterial extracts is rapidly fatal.

An organ abscess model has provided greater insight into the contribution of sortase A to the pathogenesis of staphylococcal disease. Following injection of a sublethal dose of 10^6 CFU of *S. aureus* strain Newman into the bloodstream, about 1 to 2 log units of staphylococci are rapidly killed by phagocytic cells (112). Those microbes that escape phagocytosis by adherence to specific tissues or invasion of cells can seed abscesses in virtually all organ tissues of mice (104). Abscesses mature within 4 to 5 days and harbor several log units of viable staphylococci, which are then cleared over a period of 5 to 10 days (3). Removal of organ tissue from infected animals and anatomical analysis or enumeration of viable staphylococci can be used as a measure of virulence and pathogenesis. Compared to the wild-type parent strain Newman, *srtA* mutants display a 3-log-unit reduction in bacterial growth within abscesses in multiple different organs, consistent with the notion that surface proteins of staphylococci are required to resist phagocytic clearance and to escape innate immune responses by directing bacteria to various organ tissues (119).

The septic arthritis model was developed by Bremell et al. (15, 16). Following intravenous injection, staphylococci replicate in joints, causing infectious arthritis, bone destruction, and deformation during wound healing in addition to weight loss. The severity of the infectious arthritis can be quantified by analyzing pathological anatomical lesions after excision of joints. Again sortase A mutants displayed a large reduction in virulence in this animal model system (87, 88).

Staphylococcal endocarditis occurs mainly as infectious foci on heart valves, and damaged valve tissue with fibrin-covered lesions represent a risk factor. This important clinical infection can be recapitulated in rats by first introducing valve tissue lesions with fibrin and platelet deposits via an intravenous polyethylene catheter (130). After the catheter is implanted, animals are challenged with staphylococcal infection, which causes formation of infectious thrombi and deposits of staphylococci on valve lesions followed by tissue destruction. Two days after infection, the hearts are aseptically removed and bacterial titers are determined as CFU. In this experiment, *srtA* mutants displayed a 2-log-unit reduction in virulence compared with the wild-type parent strain *S. aureus* Newman (213).

The complete spectrum of molecular mechanisms whereby surface proteins contribute to the pathogenesis of *S. aureus* infectious diseases cannot yet be appreciated. In fact, only recently have we learned about the contribution of these few surface proteins to pathogenesis, and much work is required to gain a better understanding. Nevertheless, the overall contribution of these surface molecules to staphylococcal pathogenesis can be measured by comparing wild-type and *srtA* mutant strains in infectious disease models. As is reviewed in detail above, *srtA* is a key virulence factor of staphylococci. In light of the rising number of antibiotic-resistant *S. aureus* strains (13), the sortase enzyme has become an important target for the treatment of staphylococcal disease. Additionally, surface proteins must be considered for therapeutic and preventive strategies to combat the tide of infections with this microbe.

Sortase A Structure

Sortase A harbors an N-terminal hydrophobic segment that functions as a signal peptide for secretion and as a stop transfer signal for membrane anchoring. Membrane localization of sortase was confirmed experimentally after immunoblot analysis of *S. aureus* subcellular fractions (119). The enzyme adopts a type II membrane topology, with the N terminus inside the cytoplasm and the C-terminal enzymatic portion located across the plasma membrane. Sortase A is a founding member of this

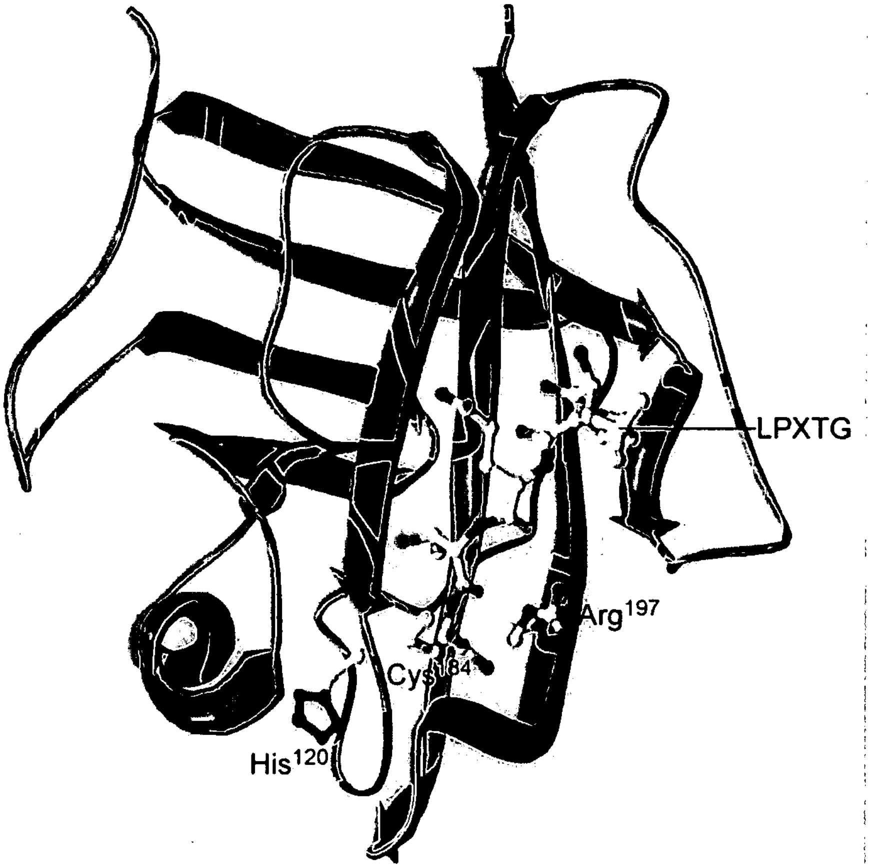

FIG. 4. Structure of *S. aureus* sortase A bound to the LPETG substrate. Sortase folds into an eight-stranded β-barrel structure. The active site resides in a depression formed by β7 and β8 strands. The side chains of His^{120}, Cys^{184}, and Arg^{197}, all of which are absolutely conserved among sortases and are required for activity, as well as the LPETG substrate are drawn with ball-and-stick structures. Cys^{184} performs a nucleophilic attack on the peptide bond between the threonine and the glycine residues of the substrate, resulting in the formation of an acyl intermediate with the carboxyl group of the C-terminal threonine thioester linked to the sulfur of Cys^{184}. This intermediate is resolved by a second nucleophilic attack on the thioester bond, which results in the release of the reaction products (the structure was generated from atomic coordinates deposited in Protein Data Bank, PDB ID 1T2P) (227).

family of sortases (84, 199). A second group of sortase-like gene products (see below) harbor an N-terminal signal peptide and a C-terminal membrane anchor, and these enzymes are thought to assume a type I membrane topology, with the N-terminal enzymatic portion projecting towards the bacterial surface and the C-terminal end residing in the cytoplasm.

In order to obtain soluble enzyme for in vitro activity assays and structural analysis, the N-terminal signal peptide/membrane anchor of sortase A was replaced with a six-histidyl tag and recombinant protein was purified (84, 197). Preliminary examination of the NOESY (*n*uclear *O*verhauser *e*ffect *s*pectroscop*y*) signals of sortase nuclear magnetic resonance (NMR) spectra suggested that the enzyme folds into a predominantly β-strand structure (83). This conjecture was corroborated by determining the three-dimensional structure of sortase by NMR spectroscopy (84) and X-ray crystallography (227). The enzyme assumes a unique fold, consisting of an eight-stranded β-barrel that includes one or two helices and several loops (Fig. 4). Strands β7 and β8 form the floor of a hydrophobic depression where the active site is located. The NMR structure showed that the absolutely conserved Cys^{184} and His^{120} residues of sortases reside within the active site (84). While Cys^{184} is anchored in β7, His^{120} is located within a helical region that connects β2 and β3, with its imidazole group in the vicinity of the sulfhydryl side chain of Cys^{184}. The NMR structure showed Asn^{98} anchored at the C-terminal end of β4 and also protruding near the active site. Asn^{98} is only poorly conserved among sortases. Further, all three aforementioned residues were positioned in a configuration similar to that of the Cys^{25}-His^{159}-Asn^{175} triad of cysteine proteases in the papain family (84, 210). X-ray crystallography data suggest, however, that Asn^{98} and His^{120} are not in the same close proximity as is observed for papain-type proteases and that sortase-mediated catalysis at Cys^{184} may occur by another mechanism (227). Arg^{197}, anchored in β8, is located in close proximity and parallel to the active-site cysteine (227) (see below). The significance of these structural observations was addressed by measuring the activity of mutant enzymes bearing alanine substitutions of critical residues (see below). Replacement of either Cys^{184} or His^{120} completely abolished sortase activity both in vivo and in vitro (197, 200, 201), and replacement of Arg^{197} greatly reduced the enzymatic activity (116). In contrast, replacement of Asn^{98} with alanine had no effect on sortase activity (116).

High-resolution X-ray structure data of sortase bound to LPETG peptide provided insight into the molecular interaction between the enzyme and its bound substrate (227). The substrate binding site resides in a concave plane molded by the β7 and β8 strands, and the scissile peptide bond between threonine and glycine is positioned between the side chains of Cys^{184} and Arg^{197} (Fig. 4). It seems plausible that sortase employs a cysteine-arginine dyad; i.e., arginine may function as a base for thiol ionization during catalysis (225, 228). Leucine and proline residues of the LPETG peptide are bound in the C-terminal region of β7, surrounded by several highly hydrophobic residues (228). NMR analysis of the ^{1}H-^{15}N chemical shifts of sortase in the presence or absence of ligand allowed identification of residues that comprise the LPXTG binding surface (108). Residues perturbed after ligand binding also mapped to the C-terminal region of the β7 strand (Thr^{180} and Ile^{182}) and to the vicinity of the loop connecting strands β3 and β4 (Ala^{118}). Importantly, Thr^{180} and Ala^{118} are absolutely conserved and Ile^{182} is partially conserved among sortases. Mutation of these residues significantly impaired sortase activity in vitro (108).

In the NMR structure, the β3-β4 and β6-β7 loops contain a set of acidic residues involved in calcium binding (84, 131). This cation, present in millimolar amounts in host tissues, activates sortase activity eightfold (84). Analysis of the sortase NMR spectra in the presence and absence of calcium revealed that Glu^{105}, Glu^{108}, and Asp^{108} side chains of the β3-β4 loop interact with the cation. In contrast, the β6-β7 loop forms a flap that is disordered in the absence of calcium (131, 227). As a result of metal binding, slow-motion conformational changes were detected by which Glu^{171}, positioned in the β6-β7 loop, transiently interacts with calcium and drives the flap to a closed state (131). This motion primarily affects the wall of the groove that forms the active site, which adopts a conformation better suited for the binding of the LPXTG peptide. Therefore, the binding of calcium ions activates sortase by a mechanism that may facilitate substrate binding (84, 131).

Biochemistry of the Sortase A Reaction

Purified recombinant sortase with a six-histidyl affinity tag replacement of the N-terminal membrane anchor, $SrtA_{\Delta N}$, cleaves LPETG peptide in vitro between the threonine and the glycine residues. Fluorescence resonance emission transmission (FRET) substrates, with fluorophore/quencher pairs 2-aminobenzoyl/2,4-dinitrophenyl or 5-[(2-aminoethyl)amino]naphtalene-1-sulfonyl/4-(4-dimethylaminophenyl-azo)benzoyl groups tethered to LPETG peptide, permit measurements of the sortase reaction as an increase in fluorescence due to substrate cleavage separating the fluorophore from the quencher (197, 201). Longer LPETG peptides would most likely improve substrate cleavage. However, the concomitant decrease in FRET due to the physical separation of functional groups diminishes the usefulness of such substrates. The addition of peptidoglycan substrates to the sortase reaction mixture stimulates cleavage of LPETG peptide and results in amide bond formation between the carboxyl group of threonine and the amino group of glycine in peptidoglycan cross bridges. Glycine, Gly_2, Gly_3, Gly_4, and Gly_5 all function as in vitro substrates; however, longer cross bridges display better substrate properties for the sortase-catalyzed transpeptidation reaction (197). Consistent with the notion that sortase functions as a transpeptidase in vivo, the velocity of the in vitro transpeptidation reaction with peptidoglycan is greater than the velocity of the hydrolysis reaction in the absence of cell wall substrate.

Sortase activity can be assessed in vivo by following the maturation of pulse-labeled surface protein, for example, the Seb-Spa_{CWS} reporter (202). Three species can be distinguished after labeling with [^{35}S]methionine: the full-length precursor (P1); the P2 intermediate, with cleaved a N-terminal signal peptide but still harboring the C-terminal sorting signal; and the mature (M) anchored polypeptide, in which the N-terminal signal peptide and the C-terminal sorting signal have been removed (see below and Fig. 5). The P2/M ratio is a measure of in vivo sortase activity. Using a *srtA* mutant strain and plasmids encoding sortase variants with amino acid substitutions, the contributions of individual amino acids to in vivo catalysis can be determined (116, 201).

Even before sortase had been purified, the in vivo assay was used to demonstrate that the enzyme forms an acyl intermediate with cleaved surface protein. Surface protein anchoring can be inhibited with [2-(trimethylammonium)ethyl]methanethiosulfonate (MTSET) and *p*-hydroxymercuribenzoic acid (202). This suggested that the enzyme requires a cysteine residue to catalyze the transpeptidation reaction, as methanethiosulfonate and organic mercurials react with sulfhydryl groups (2). In fact, MTSET inhibition can be rescued with dithiothreitol (DTT), which reduces the disulfide between the active-site cysteine and MTSET, thereby regenerating enzyme sulfhydryl (197). *S. aureus* sortase A harbors only one cysteine residue, Cys^{184}, which is absolutely conserved in all sortases. Replacement of Cys^{184} with alanine completely abolishes all sortase activity both in vivo and in vitro (197, 200, 201). Addition of the strong nucleophile hydroxylamine to staphylococci results in the release of surface protein into the extracellular medium. Purification and biochemical characterization of such released products revealed threonine hydroxamate at the C-terminal end of surface proteins. Hydroxylaminolysis of surface protein occurs only in the presence of sortase and absolutely requires its active-site cysteine residue. The most likely explanation for these findings is that hydroxylamine attacks the thioester between the C-terminal threonine of cleaved surface proteins and the active-site cysteine of sortase. This acyl enzyme intermediate could indeed be detected in vitro (77). Sortase was incubated with LPETG peptide and catalysis quenched by the addition of trifluoroacetic acid. Electrospray ionization mass spectrometry revealed the presence of species in which LPET peptide was tethered to the active site cysteine. These data support a mechanistic model in which Cys^{184} performs nucleophilic attack on the scissile peptide bond between threonine and glycine of the LPXTG motif (acylation step) (197). The acyl intermediate is then resolved by the nucleophilic attack of the amino group of the pentaglycine cross bridge, thereby regenerating the enzyme active site and tethering surface protein to cell wall fragments (deacylation step). These reactions are as follows: R_1-LPXT(CO-NH)-G-R_2 + E-SH↔ R_1-LPXT(CO-S)-E + NH_2-G-R_2 (acylation step) and

R_1-LPXT(CO-S)-E + NH_2-Gly_5-R_3→R_1-LPXT(CO-NH)-Gly_5-R_3 + E-SH (deacylation step).

Analysis of the kinetic parameters of the transpeptidation reaction indicates that it may resemble a ping-pong mechanism, whereby the binding and cleavage of the LPXTG is followed by the incorporation of the pentaglycine substrate into the active site for the separation of the acyl intermediate (77, 200). Each of these reactions appears to harbor a distinct limiting step, with that of the acylation step during transpeptidation and that of the deacylation step during hydrolysis (77).

The mechanism whereby Cys^{184} performs nucleophilic attack at the scissile peptide bond is not yet clear. Reagents that specifically react with sulfhydryl but not with thiolate groups such as iodoacetamide and iodoacetic acid do not inhibit sortase (202), consistent with the notion that the sortase sulfhydryl must be ionized. The NMR structure of the enzyme showed the presence of a histidine residue (His^{120}) (see above) located in the active site of the enzyme (84). The residue is absolutely conserved and essential for sortase activity, both in vivo and in vitro (201). This result prompted the hypothesis that sortase would form an imidazolium-thiolate ion pair, mimicking active-site ionization of cysteine proteases (186). In this model, the positively charged imidazol group of His^{120} stabilizes the formation of a thiolate in Cys^{184} and acts as a proton donor/acceptor during acylation and deacylation steps (201). In papain, the Cys^{25}-His^{159}-Asn^{175} triad comprises the active site (210). While cysteine and histidine form a thiolate-imidazolium ion pair that is fundamental for papain catalysis, the asparagine side chain positions His^{159} in a favorable orientation towards Cys^{25} through hydrogen bonding. In sortase, two residues, Trp^{194} and Asn^{98}, that could play a role similar to that of Asn^{175} are positioned near His^{120}; however, these amino acids are not conserved among sortases. While the replacement of Asn^{98} with alanine or glutamine does not affect sortase activity (116), mutation of Trp^{194} to alanine reduced the enzyme's activity both in vitro and in vivo (201). Thus, Trp^{194} could play a role in positioning His^{120} in the proper orientation to achieve catalysis.

The observed pK_as for the side chains of both Cys^{184} and His^{120} preclude the possibility of a thiolate-imidazolium ion pair within the sortase active site (32). Using an inhibitor of sortase obtained after the replacement of the T-G peptide bond with a vinyl sulfone, which reacts with cysteine thiolate, the investigators examined inhibition as a function of proton concentration. While the K_i, a value that reflects the binding of the inhibitor to the enzyme, remained constant, the k_i, a measure of the effectiveness of the inhibitor, increased only beyond pH 9.4 (32). This argues in favor of the presence of a thiol group in the sortase active site at physiological pH. The pK_a for the imidazol group of His^{120} was determined by NMR following the chemical shifts of ^{1}H-ε1 and ^{1}H-δ1 atoms of this residue as a function of pH. The titration suggested a pK_a of approximately 7.0 (32). Again, this indicates that at pH 7.5 the imidazol group of His^{120} would be only partially protonated. Moreover, the observed pK_a is independent of Cys^{184}, as the titration curve for a sortase Cys^{184}Ala mutant did not change (32). Together these experiments suggest that sortase catalysis cannot occur via a mechanism involving the thiolate-imidazolium ion pair, as originally proposed (84, 201).

Analysis of the X-ray crystallographic structure of sortase A with LPETG peptide led to the formulation of a new hypothesis. As is pointed out above, this structure revealed the presence of Arg^{197} in the active site (227). This residue is absolutely conserved among sortases and is positioned in front of and parallel to Cys^{184}. Replacement of Arg^{197} with alanine, lysine, or histidine greatly impaired sortase activity, both in vivo and in vitro (116). Because the guanidinium group of Arg^{197} interacts with the carbonyl group of the scissile bond in the X-ray structure, it was proposed that Arg^{197} forms an oxyanion hole that may stabilize the acylated adduct (227). This hypothesis was corroborated by an experiment in which hydroxylamine was unable to resolve the acyl intermediate when Arg^{197} was replaced by alanine or lysine, indicating that in the absence of the guanidinium group, the thioacyl intermediate is not formed (116). These results suggest that the sortase active site may comprise a cysteine-arginine dyad (225, 228). It is important to note that sortases display absolute conservation of several residues. Two of these, Leu^{97} and Tyr^{153}, have been replaced by alanine in order to assess their importance for the enzyme's activity. Despite their conservation, these residues were not required for sortase activity either in vitro or in vivo (201). The contribution of other conserved amino acids to sortase catalysis remains unknown.

The specificity of sortase A for different pentapeptide motifs was studied by determining the in vitro activity of the enzyme towards a peptide library with 18 amino acid substitutions in every position (99). This study confirmed bioinformatic analysis of sortase substrates, which indicate that the enzyme recognizes LPXTG sequences. Not surprisingly, initial-velocity analysis showed that only leucine is tolerated in position 1 in XPETG peptides and only proline is tolerated in position 2 in LXETG peptides, whereas any residue is tolerated in position 3 in LPXTG peptides. Only threonine in position 4 in LPEXG peptides is accepted as a substrate, and only glycine is accepted in position 5 in the LPETX peptide library. The enzyme's residues involved in this specificity were detected by comparing NMR signals of bound versus unbound sortase (see above) (108). Besides those in Cys^{184} and Arg^{197}, chemical shift changes in Thr^{180} and Ala^{118} (absolutely conserved residues) and Ile^{182} (partially conserved) were also detected. Mutation of these residues significantly impaired sortase activity in vitro (108). Whether these residues contribute to the substrate specificity of sortase remains to be assessed, and it would be interesting to screen the peptide library and determine whether peptides with sequences differing from LPXTG can be substrates of these mutants.

Another important aspect of the sortase reaction is the interaction of the enzyme with its cell wall substrate, the pentaglycine cross bridge. In vivo, sortase can catalyze the transpeptidation of surface proteins to cell wall cross bridges containing one, three, and five glycine residues, but not to the ε-NH_2 group of the L-lysine residue of wall peptides. This conclusion was reached following analysis of the anchor structure of surface proteins generated by *S. aureus fem* mutants defective in cross bridge biosynthesis (196). At least three Fem factors (*f*actors *e*ssential for *m*ethicillin resistance) are required for the addition of glycine residues to the cross bridge of *S. aureus* peptidoglycan (101). FemX is responsible for the addition of the first glycine residue to the L-lysine of the wall peptide, while

FemA adds the second and third glycine residues and FemB completes the cross bridge by incorporating the fourth and fifth glycine. Therefore, *femB* mutants synthesize Gly_3 cross bridges, *femA* mutants synthesize Gly_1 cross bridges, and a partial *femAX* mutant either carries Gly_3 cross bridges or completely lacks cross bridge (97). The cell wall anchor structure of Φ11-hydrolase-released $Seb\text{-}Spa_{CWS}$, which is expressed in each of these *fem* mutants (see above), revealed that sortase can link surface protein to Gly_5, Gly_3, and Gly_1 cross bridges in wild-type, *femB*, *femA*, and *femAX* strains but failed to anchor protein to the ε-amino of L-Lys (196). Nevertheless, the velocity of the sorting reaction is diminished in *fem* mutants compared to the wild type (196), indicating that sortase prefers pentaglycine as a cell wall substrate. This conjecture was corroborated in vitro by the observation that Gly, Gly_2, and Gly_3 can be used as nucleophiles by the enzyme and are linked to the threonine of LPETG peptides (77, 200). Diglycyl-histidine and diglycyl-leucine can also be used in the in vitro transpeptidation reaction, although the binding is decreased (as deduced from the apparent K_m values). Glycyl-alanine and glycyl-valine also retain substrate properties, but their binding is reduced by 10-fold. In contrast, alanyl-glycine and valyl-alanine cannot be used as substrates for the transpeptidation reaction (77). Thus, cell wall substrate recognition of sortase tolerates only glycine as the N-terminal residue and strongly prefers another glycine at the second position. While the enzyme's constraints for the selection of a cell wall substrate are being delineated, the actual binding site for peptidoglycan substrate remains unknown. Gly_3 substrate was modeled into the crystal structure of sortase (227). It was speculated that Gly_3 may be positioned in the loop that connects β7 and β8, replacing a water molecule that otherwise contacts the backbone atoms of this loop. Nevertheless, experimental data are needed to reveal the peptidoglycan binding site of sortase.

Lipid II, the Peptidoglycan Substrate of Sortase A

Cell wall active-antibiotics have been employed to probe the peptidoglycan substrate requirements for the sortase reaction (197). Vancomycin binds D-Ala–D-Ala within lipid II and inhibits the transglycosylation and transpeptidation reactions that assemble peptidoglycan from this precursor (193, 211). Moenomycin is a lipid II analog that interferes with the transglycosylation reaction of peptidoglycan biosynthesis (168, 207). Lastly, penicillin inhibits only the transpeptidation reaction by occupying the corresponding active sites of PBPs without affecting transglycosylation or lipid II concentrations (188, 193). By measuring cell wall anchoring of pulse-labeled reporter proteins, it was shown that both vancomycin and moenomycin, but not penicillin G, interfered with the cell wall sorting pathway (202). As the inhibition of surface protein anchoring increased during prolonged incubation of staphylococci with vancomycin or moenomycin, a plausible explanation for these results is that antibiotics reduce the availability of lipid II, which serves also as the peptidoglycan substrate of sortase A.

Additional evidence for lipid II as the peptidoglycan substrate for surface protein anchoring was garnered with in vitro reactions. LPXTG peptide is linked to lipid II by purified sortase A, and vancomycin can block this reaction (165). Analysis of surface protein anchoring in protoplasts promoted the notion that the sorting reaction does not require mature, assembled peptidoglycan (202). The cell wall envelope of *S. aureus* was removed by digestion with muralytic enzyme, protoplasts were pulse-labeled with $[^{35}S]$methionine, and the radiolabeled surface protein was immunoprecipitated. Protoplasts catalyzed surface protein precursor cleavage at the LPXTG motif at a rate similar to that for staphylococci with intact cell wall envelopes. A unique surface protein sorting intermediate was detected in protoplast membranes. Further evidence for a linkage between surface proteins and lipid II in vivo was obtained by labeling staphylococci with $[^{32}P]$phosphoric acid, which is incorporated into lipid II molecules (154). Following removal of the cell wall envelope with muramidase, which cannot cleave lipid II, labeled polypeptides were immunoprecipitated and detected by autoradiography. ^{32}P-labeled surface protein species were identified, and their synthesis required sortase A activity. Radiolabeled lipid II could be removed from surface protein by lysostaphin cleavage at pentaglycine cross bridges, whereas muramidase, which cannot cleave lipid II, displayed no effect. Treatment of staphylococci with tunicamycin, an inhibitor of phosphor-*N*-acetylmuramyl-pentapeptide translocase (the enzyme required for formation of lipid I and lipid II [191]) abolished sortase A-dependent biosynthesis of ^{32}P-labeled surface protein. The C-terminal anchor of immunoprecipitated ^{32}P-labeled surface protein was analyzed by thin-layer chromatography and observed to bind nisin (154), an antibiotic that specifically interacts with lipid II (214). Thus, the cell wall sorting intermediate P3 is comprised of surface protein linked to lipid II (154). A model that emerged from these studies suggests that P3 not only is the product of the sortase reaction but also serves as a substrate for the transglycosylation and transpeptidation reactions of cell wall biosynthesis, similar to the case for lipid II (Fig. 5). Obviously, the amino group of the pentaglycine cross bridge of P3 is already engaged in an amide bond and cannot perform the nucleophilic attack at PBP acyl enzyme intermediates with cleaved wall peptides. Nevertheless, the pentapeptide structure permits PBP cleavage at the D-Ala–D-Ala of P3 and attachment of other pentaglycine cross bridges from neighboring wall peptides at this site. In this manner, the P3 sorting intermediate can be fully incorporated into the three-dimensional network of staphylococcal peptidoglycan.

Sortase A Inhibitors

Inhibitors of sortase should be useful for the characterization of this fascinating enzyme. However, can such inhibitors affect the outcome of human or animal infection with *S. aureus*? If virulence studies with *srtA* mutants provide a correlate for the contribution of sortase A to disease, we can be hopeful that inhibitors of the sortase reaction may display therapeutic effects. Moreover, as sortase is a universal virulence factor of gram-positive pathogens, compounds that inhibit the enzyme's activity could constitute antimicrobial agents for the treatment of many diseases, such as enterococcal and pneumococcal infections. Until such specific sortase inhibitors have been isolated and tested, it is impossible to say whether this anti-infective strategy will be inferior or equal to that of conventional antibiotic therapy. Certainly, there is no precedent for use of clinically relevant anti-infectives, i.e., inhibitors

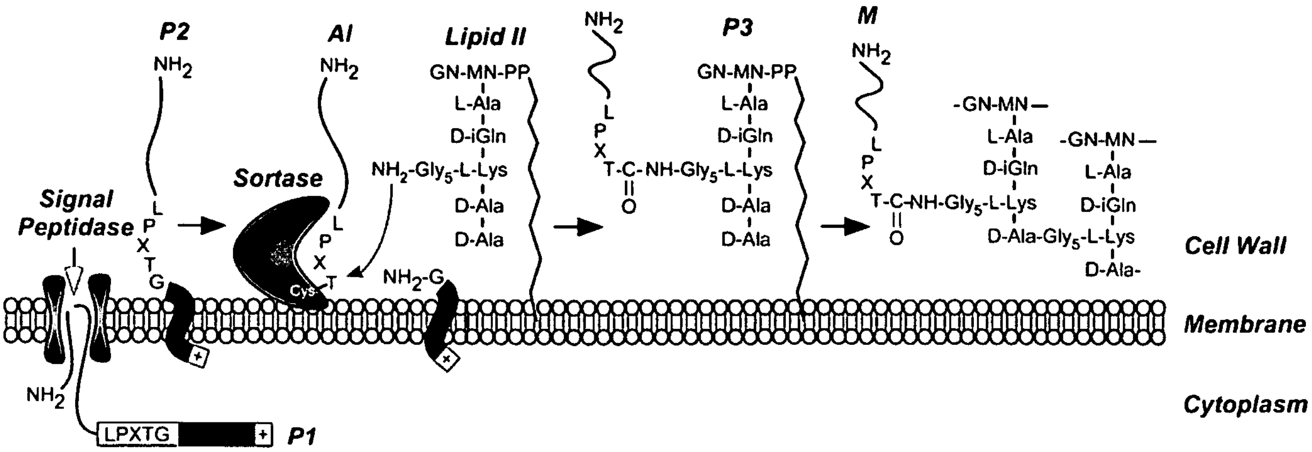

FIG. 5. Cell wall sorting pathway of surface proteins in gram-positive bacteria. Surface proteins are first synthesized in the bacterial cytoplasm as full-length precursors (P1) containing an N-terminal signal sequence and a C-terminal sorting signal. The signal sequence directs the cellular export of the polypeptide through the Sec system and, upon translocation, is cleaved by signal peptidase. The product of this reaction, the P2 precursor harboring only the C-terminal sorting signal, is retained within the secretory pathway via its C-terminal hydrophobic domain (black box) and positively charged tail (+). Sortase, a membrane-anchored transpeptidase with active-site cysteine, cleaves the peptide bond between the threonine (T) and the glycine (G) of the LPXTG motif, generating an acyl intermediate (AI). Lipid II, the peptidoglycan biosynthesis precursor, and its pentaglycine cross bridge (Gly_5) amino group attack the acyl intermediate, linking the C-terminal threonine of the surface protein to lipid II (P3 precursor) and regenerating the active site of sortase. The P3 precursor functions as a substrate for penicillin binding proteins and is incorporated into the cell wall envelope to generate mature anchored surface protein (M), which is also displayed on the bacterial surface. This pathway is universal in many gram-positive bacteria, and the functional elements of cell wall cross bridges, LPXTG motif, sortase, and penicillin binding proteins are conserved.

of bacterial virulence factors, as a therapeutic strategy for human infectious diseases. These thoughts should not distract us from the pressing need for the development of new therapeutic agents, as staphylococci have developed mechanisms of resistance to all known antibiotics, including methicillin and vancomycin (14).

The first search for sortase inhibitors occurred even before the enzyme was identified (202). Methane-thiosulfonates such as MTSET and (2-sulfonatoethyl)methane-thiosulfonate inhibit sortase in vivo and in vitro, with MTSET achieving complete inhibition. The mercurial *p*-hydroxymercuribenzoic acid could also inhibit sortase. All of these compounds react with the catalytic Cys^{184} and prevent formation of acyl intermediates. In contrast, sulfhydryl alkylating agents such as iodoacetamide, *N*-ethylmaleimide, or iodoacetic acid do not inhibit sortase. While these reagents proved useful to elucidate the catalytic mechanism of the enzyme, nondiscriminate interactions of thiol-reactive molecules renders these compounds useless for therapeutic studies because of their associated toxicity in mammalian organisms.

Several recent efforts have examined natural or chemical compounds for the property of inhibiting sortase A in vitro. For example, extracts from 80 medicinal plants were tested and those obtained from *Cocculus trilobus*, *Fritillaria verticillata*, *Liriope platyphylla*, and *Rhus verniciflua* displayed inhibitory activity (95). The extract from *Fritillaria verticillata* bulbs was subjected to silica gel chromatography, and a fraction with potent inhibitory effects on sortase was isolated. The constituent of this fraction was identified by NMR as glucosylsterol β-sitosterol-3-*O*-glucopyranol (93). As sitosterol alone does not inhibit sortase, it was concluded that the inhibitory effect must reside within the glucopyranoside moiety of the molecule. A similar experimental approach for extracts of *Coptis chinensis* identified the isoquinoline alkaloid berberine chloride as a sortase inhibitor (94). Both compounds exhibit a lower MIC than *p*-hydroxymercuribenzoic acid (see above) and were able to inhibit binding of *S. aureus* to fibronectin-coated surfaces (143), an interaction mediated by the sortase A substrates fibronectin binding proteins A and B (FnbpA and FnbpB) (see above). However, the k_i values for these inhibitors have not been obtained, precluding their comparison with other known sortase inhibitors.

Another strategy for the development of inhibitors employed modifications to the scissile bond of LPXTG peptides. In the first of these studies, the threonine-glycine peptide bond was substituted by moieties known to alkylate the active-site thiol of cysteine proteases. These included peptidyl-diazomethane (LPAT-CHN_2) and peptidyl-chloromethane (LPAT-CH_2Cl) (176). Both compounds successfully inhibited sortase activity in vitro, with a k_i/K_i of 2.2×10^4 $M^{-1} \cdot min^{-1}$ ($k_i = 5.8 \times 10^{-3}$ min^{-1}) for LPAT-CHN_2 and a k_i/K_i of 2.1×10^4 $M^{-1} \cdot min^{-1}$ ($k_i = 1.1 \times 10^{-2}$ min^{-1}) for LPAT-CH_2Cl. In a second study, the scissile bond was replaced with vinyl sulfone [LPAT-SO_2(Ph)], a moiety known to covalently modify the active-site thiolate of cysteine proteases via formation of a thioether adduct (32). Due to the requirement for ionization of the thiol group of Cys^{184}, this modified peptide achieved maximal inhibition at pHs greater than 8.0. As expected, inhibition was irreversible, and at pH 7.0 the k_i/K_i was measured to be 44.4 $M^{-1} \cdot min^{-1}$ ($k_i = 4 \times 10^{-4}$ min^{-1}). Different types of vinyl sulfones, i.e., di-, ethyl-, methyl-, and phenyl vinyl sulfones, all inhibited sortase A. Phenyl vinyl sulfone (PVS) displayed the greatest effect, with a k_i/K_i of 20.1 $M^{-1} \cdot min^{-1}$ (55). Interestingly, PVS-treated *S. aureus* cells failed to bind to a fibronectin-coated surface, suggesting that PVS can inhibit the sortase-dependent surface display of fibronectin binding proteins in vivo. However, additional studies documenting the effects of PVS on mammalian cell viability and on other steps of the sorting reaction are required for a clearer understanding and confirmation of this inhibition.

Substrate peptides have been generated with the expectation of mimicking the transition state for the formation of sortase acyl intermediates. In order to obtain such an inhibitor, the threonine residue of an LPETG peptide was replaced by a phosphinate group ($LPE\Psi\{PO_2H\text{-}CH_2\}G$) (98), a peptide modification that has been successfully used for the design of zinc protease inhibitors. As the tetravalent coordination of the phosphorous atom imitates the acyl intermediate transition state, this modified peptide should compete with LPETG substrate for the sortase active site. Inhibition was achieved with the phosphinate compound and was therefore exploited to determine different kinetic parameters of the sortase reaction (98).

Another strategy for the discovery of sortase inhibitors has been to screen libraries of small-molecule compounds (142). One thousand compounds were tested for their ability to inhibit sortase in vitro, and the initial hits were subjected to successive structural and chemical modifications with the goal of achieving more pronounced inhibitory effects on sortase activity. This resulted in the isolation of a set of substituted (*Z*)-diarylacrylonitriles that exhibit potent inhibition towards sortase. Most of the compounds described here were tested only in vitro and typically require micromolar or low millimolar concentrations for inhibition of sortase. Much work still needs to be done before one can analyze compounds with K_i at low micromolar or nanomolar concentrations and with inhibitory specificity that permits testing in animal models of *S. aureus* pathogenesis.

Applications of the Sortase A Reaction

Sortase-catalyzed transpeptidation is an attractive protein engineering tool for the incorporation of nonpeptide moieties into polypeptides tagged with an LPXTG motif. Several established modification systems make use of recombinant proteins conjugated to peptide analogs, unnatural amino acids, fluorophores, and other biochemical and biophysical probes. One strategy to achieve such modification is subtilisin-based peptide ligation (23). However, this technology involves several biosynthetic steps and is not efficient. Sortase transpeptidation, on the other hand, offers a simple and efficient tool for the incorporation of chemicals containing glycine residues with a free amino group to the LPXTG motif of recombinant proteins. As a proof of concept, triglycyl-lysine-folate was synthesized and incubated with purified recombinant green fluorescent protein (GFP)-LPETG-His_6 (i.e., GFP containing a C-terminal LPETG–six-histidyl group) in the presence of sortase (114). The products of the reaction were separated by reverse-phase high-pressure liquid chromatography and analyzed by matrix-assisted laser desorption ionization–time-of-flight analysis, revealing that the GFP-LPET-G_3K(folate) adduct was produced with high efficiency. Another biotechnological application is the incorporation of the branched peptide AT-P-022 into polypeptides. AT-P-022 possesses strong protein transduction activity; i.e., it promotes the uptake of linked proteins by eukaryotic cells. Due to its branched structure, however, it is difficult to incorporate AT-P-022 into proteins. Using sortase-mediated peptide ligation, it was possible to generate a GFP-LPET-G2(AT-P-022) conjugate in a single-step reaction. Fluorescence analysis demonstrated that the reverse-phase high-pressure liquid chromatography-purified product was taken up by NIH 3T3 cells with high efficiency (114).

Another application of the sortase reaction is the generation of self-cleavable chimeras for one-step purification of recombinant proteins (113). The concept relies on the expression and purification of a recombinant His_6-sortase-LPETG-target protein fusion that cleaves itself once the enzyme has been activated by the addition of calcium and triglycine. The transpeptidation product, i.e., nontagged target protein, can be eluted in a single chromatography step, with glycine as the only modification introduced by the purification procedure. The sortase strategy differs from other systems employing N-terminal carriers that are cleaved off from the target protein by the addition of a protease, in which the separation of the target protein from the protease requires additional chromatography steps. The approach was tested for the purification of GFP, Cre, and p27 proteins (113). In all cases, the presence of an N-terminal sortase carrier increased the expression and solubility of the recombinant protein. Importantly, neither autocleavage nor transpeptidation with *E. coli* proteins containing an N-terminal glycine was observed during expression. Following affinity chromatography of cleared cell lysate on Ni-nitrilotriacetic acid Sepharose and several washes, charged resin was incubated in buffer containing calcium and Gly_3. Concentrated and 98% pure target protein was recovered from the supernatants, indicating that sortase-based protein purification provides a simple and effective method that may be generally applicable to many proteins.

S. AUREUS SORTASE B

Sortase homologs have been revealed in every gram-positive bacterium for which genome sequences are available, and most species encode more than one sortase (148). *S. aureus* encodes two sortases, and the second enzyme has been named sortase B (122, 123). The structural gene for sortase B (*srtB*) is part of the *isd* (*i*ron-regulated *s*urface *d*eterminant) locus, which is comprised of three transcriptional units, *isdA*, *isdB*, and *isdCDEF-srtB-isdG* (Fig. 6A) (123). IsdA, IsdB, and IsdC are cell wall-anchored proteins. IsdD is thought to be inserted into the plasma membrane. IsdE lipoprotein and the IsdF ATP binding cassette (ABC) transporter presumably function as heme-iron transporters in the plasma membrane. IsdG is located in the cytoplasm, and it cleaves the heme tetrapyrrol ring and liberates iron for staphylococcal growth. IsdA, IsdB, IsdC, IsdD, IsdE, and IsdG all bind heme-iron (121). Additionally, IsdB (but not IsdA or IsdC) binds hemoglobin (121). Two additional Isd proteins are encoded elsewhere in the genome of *S. aureus*: IsdH (HarA), a haptoglobin binding, cell wall-anchored protein (42), and IsdI, an IsdG homolog with heme oxygenase activity (181). A *fur* box (46), i.e., a DNA sequence to which the *f*erric *u*ptake *r*epressor binds and inhibits transcription when staphylococci grow in iron-replete conditions (76, 215), is present in the promoter regions of all of these genes. Thus, the Isd proteins and sortase B are expressed only under conditions when iron is limiting (121, 123). However,

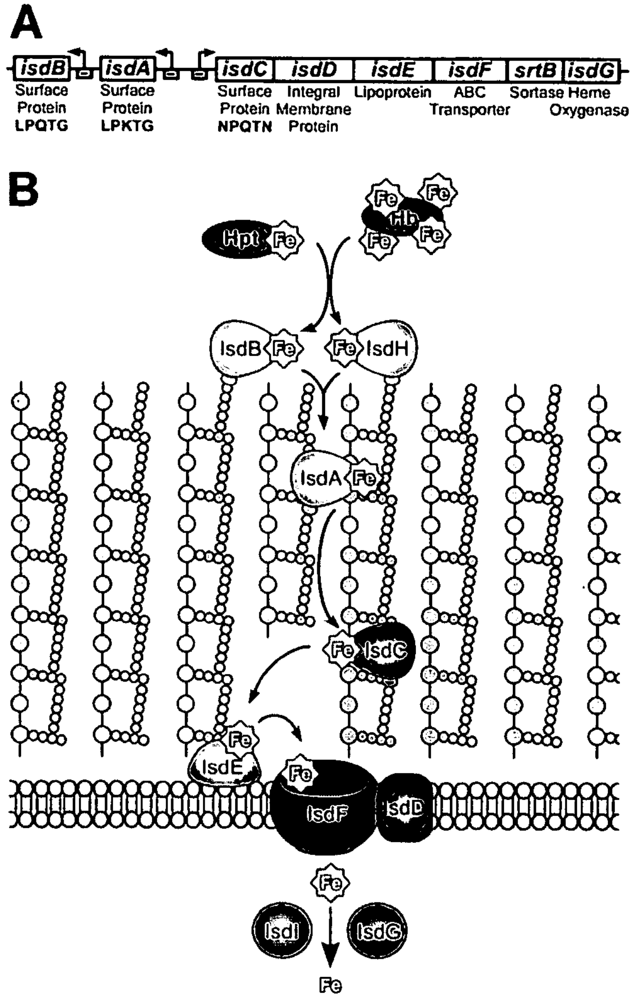

FIG. 6. Isd-mediated heme-iron uptake in *S. aureus*. A. The *isd* locus is comprised of *isdA*, *isdB*, and *isdC*, which encode cell wall-anchored proteins carrying LPKTG, LPQTG, and NPQTN motifs in their respective sorting signals. Located elsewhere in the *S. aureus* genome, *isdH* and *isdI* encode a fourth LPKTG surface protein and a heme oxygenase, respectively. All *isd* genes are regulated by the ferric uptake repressor (Fur), which represses transcription under iron-replete conditions by binding to *fur* boxes present in promoter regions (shaded boxes). Arrows indicate the direction of transcription. B. A model for Isd-mediated heme-iron transport across the cell wall of *S. aureus*. IsdA, IsdB, and IsdH are anchored to the cell wall by sortase A and function as receptors for hemoprotein ligands, including haptoglobin (Hpt), hemoglobin (Hb), or heme. Upon binding to Isd receptors, heme is released from the hemoproteins by an as-yet-undefined mechanism and passaged through the cell wall in an IsdC-dependent manner. Treatment of staphylococcal cells with extracellular proteinase K completely degrades IsdB, only partially digests IsdA, and leaves IsdC intact, suggesting different degrees of surface exposure for each of these cell wall proteins. The heme molecule is then transported through the membrane transport system composed of IsdDEF into the cytoplasm. Upon entry into the cytoplasm, heme is degraded by IsdG and IsdI heme monooxygenases. This leads to the release of free iron for use by the bacterium as a nutrient source. (Adapted from reference 182 with permission from Elsevier.)

fur mutant staphylococci express the *isd* locus and *srtB* in a constitutive fashion (123).

IsdC and Sortase B Contribute to Heme-Iron Transport

IsdC, a cell wall-anchored protein, is the only known substrate of sortase B. In contrast to IsdA, IsdB, and IsdH, each of which contains LPXTG-type sorting signals and is a substrate for sortase A, the IsdC sorting signal harbors an NPQTN motif. Cell wall anchoring of IsdC or Seb-IsdC$_{CWS}$ is abolished in a *srtB* mutant strain; however, *srtA* mutants attached both proteins to the envelope in a fashion similar to that of wild-type staphylococci (123). Deletion of the *srtB* gene did not interfere with the anchoring of 15 different surface proteins harboring LPXTG motif sorting signals. Thus, sortase B uniquely recognizes its IsdC substrate and tethers the polypeptide to the staphylococcal peptidoglycan. Unlike sortase A-anchored substrates that are displayed on the bacterial surface, cell wall-anchored IsdC remains buried within the cell wall envelope. Two lines of evidence support this conclusion. First, IsdC, but not IsdA or IsdB, is protected from digestion with extracellular protease unless the integrity of the cell wall envelope is perturbed by treatment with muralytic enzyme (121). Further, IsdA and IsdB are detectable by immunofluorescence microscopy, indicating that surface-displayed polypeptides bind to antibody. However, specific antibody added to intact staphylococci may not bind to cell wall-anchored IsdC (L. A. Marraffini and O. Schneewind, unpublished observation). Seb-Spa$_{CWS}$, carrying a C-terminal fusion of enterotoxin B to the protein A sorting signal, is a substrate for sortase A (195). Cell wall-anchored Seb-Spa$_{CWS}$ is displayed on the bacterial surface and can be degraded by proteinase K digestion. In contrast, cell wall-anchored Seb-IsdC$_{CWS}$ is not displayed on the bacterial surface and can be degraded by extracellular protease only when the integrity of the cell wall envelope is perturbed by treatment with muralytic enzymes (115). Together these experiments demonstrate that sortase A and sortase B target their protein substrate to discrete locations within the cell wall envelope. Further, the information for the ultimate destination of a polypeptide in the staphylococcal envelope resides within its cell wall sorting signal. Proper targeting to discrete locations in the cell wall envelope requires polypeptide substrate interactions with cognate transpeptidases and specific yet distinct peptidoglycan substrates for each of the two sortases (115).

What is the purpose of anchoring IsdC at a discrete site within the cell wall envelope? A plausible explanation is that sortase A-anchored proteins, i.e., IsdA, IsdB, and IsdH, capture hemoproteins on the bacterial surface and dislodge heme from host polypeptides (Fig. 6B). Transfer of heme from sortase A-anchored polypeptides to sortase B-anchored IsdC in the cell wall envelope, followed by subsequent transfer of heme-iron to IsdD and IsdEF, is thought to provide for the passage of this essential nutrient across the 100-nm-thick cell wall envelope. Once transported across the plasma membrane, iron may be released from heme via IsdG- or IsdI-mediated tetrapyrrol cleavage (182).

The contribution of sortase B to heme-iron uptake was examined in *srtB* mutant staphylococci. Growth media were depleted of divalent cations and supplemented with heme-iron.

While wild-type staphylococci were able to grow under these conditions, *srtB* mutant bacteria were not (121). Although *S. aureus* is capable of synthesizing heme-iron both in the presence and in the absence of *srtB*, only uptake of exogenous heme-iron was affected by deletion of the *srtB* gene. Measurement of the uptake of [^{55}Fe]heme in wild-type and *srtB* mutant staphylococci with intact cell wall envelopes or in osmotically stabilized protoplasts showed that *srtB* is required for heme-iron uptake under both conditions (121). Thus, the lack of sortase B activity or the absence of anchored IsdC not only prevents the passage of heme-iron across the cell wall envelope but also prevents heme-iron transport across the plasma membrane into the bacterial cytoplasm.

Molecular Genetic Analysis of Sortase B (*srtB*) Function

Iron is an essential nutrient for most microbes, including staphylococci, and although iron is abundantly present in host tissues, its availability is severely restricted by sequestering iron via bound proteins and cellular compartments (17). To test whether sortase B is required for the pathogenesis of staphylococcal infections, virulence properties of *srtB* and *srtA* mutant staphylococci were compared with those of the wild-type parent strain *S. aureus* Newman. The calculated LD$_{50}$ after intraperitoneal injection of a *srtB* mutant was not significantly different from that of wild-type *S. aureus*, indicating that sortase B is dispensable during acute early phases of infection (213). In the renal abscess model following intravenous infections of mice, isogenic *S. aureus* variants carrying a *srtB* deletion displayed a small defect in virulence, which became more pronounced during later stages of infection (123, 213). Using the rat infectious endocarditis model, no difference was observed in the number of wild-type or *srtB* mutant staphylococci multiplying on cardiac vegetations (213). Measurement of the arthritic index as well as the number of staphylococci present in the joints during the murine infectious arthritis model demonstrated that the *srtB* mutant is significantly less virulent than the wild-type strain (88, 213). The *srtB* defect is not as pronounced as that of *srtA* variants; however, in double mutant strains the *srtA srtB* deletions caused an additive defect in virulence compared to that of the single mutant strains (88, 213). Together these results revealed the contributions of sortase B to *S. aureus* pathogenesis, in particular to infections that require bacterial persistence in host tissues. The contribution of sortase B is additive to that of sortase A, indicating that the two enzymes perform nonredundant and complementary functions and that each promotes the establishment of staphylococcal disease.

Biochemistry of the Sortase B Reaction

Recombinant sortase B has been purified, and, as reported for sortase A, the N-terminal membrane anchor sequence was removed to obtain soluble enzyme. SrtB$_{\Delta N}$ cleaves NPQTN peptide substrate but not LPETG peptides (123). The enzyme is inhibited with MTSET and this inhibition can be relieved with DTT, indicating that sortase B also utilizes its sole cysteine residue (Cys^{223}) for catalysis. In contrast to that of sortase A, sortase B activity is very low, which has thus far precluded a detailed biochemical analysis of its transpeptidation reaction (123). To reveal the site of cleavage at the IsdC substrate, i.e., the NPQTN motif, and to examine the peptidoglycan substrate for sortase B, the cell wall anchor structure of IsdC was determined. An engineered reporter protein, Seb-MHis$_6$-IsdC$_{CWS}$, is anchored to the cell wall envelope in a fashion similar to that for IsdC. After the cell wall envelope of *S. aureus* expressing the reporter was cleaved with lysostaphin (169), the polypeptide was purified and cleaved with cyanogen bromide, and C-terminal anchor peptides were purified by a second round of affinity chromatography. Matrix-assisted laser desorption ionization–time-of-flight (MALDI-TOF) analysis of anchor peptides revealed that sortase B cleaves NPQTN motif sorting signals between the threonine and the asparagine residues. The C-terminal threonine residue is amide linked to the amino groups of pentaglycine cross bridges within the staphylococcal cell wall (115). Thus, the chemical product of the sortase B reaction has striking similarity to that of the sortase A reaction. Nevertheless, detailed analysis of peptidoglycan treated with mutanolysin (21, 224) or Φ11 hydrolase (137) revealed only a very limited degree of cross-linking between IsdC anchor peptides compared to that between the anchor peptides generated by sortase A (115, 137). About 80 to 95% of all murein subunits of assembled peptidoglycan harbor cell wall tetrapeptides with cross-linked D-Ala at position four (57, 184, 188), and this can also be observed for sortase A-anchored surface proteins, which are embedded at any position in glycan chains with up to 11 MurNAc-GlcNAc disaccharide units and cross-linked to as many as 15 cell wall peptides (137). In contrast, sortase B-anchored product is attached to at most six or seven disaccharide subunits, and its wall peptides are either non-cross-linked (murein petapeptides) or linked to two or three peptidoglycan subunits (Fig. 7).

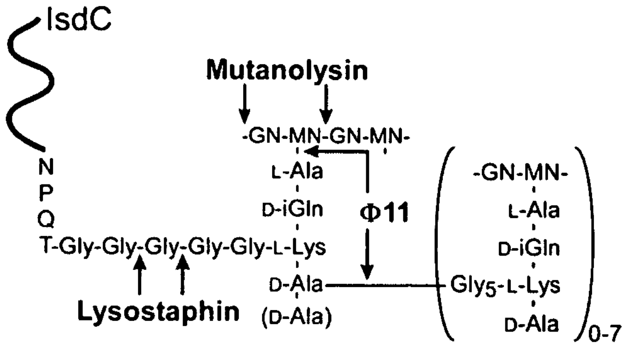

FIG. 7. Cell wall anchor structure of staphylococcal IsdC. The C-terminal threonine of IsdC, generated by sortase B-mediated cleavage between the threonine and the asparagine of the NPQTN motif, is amide linked to the pentaglycine cross bridge of *S. aureus* cell wall peptidoglycan. Treatment of the staphylococcal peptidoglycan with lysostaphin (glycyl-glycine endopeptidase), mutanolysin [*N*-acetylmuramidase that cleaves the β(1-4) *O*-glycosidic bond between *N*-acetylmuramic acid and *N*-acetylglucosamine (GN)], amidase (*N*-acetylmuramoyl-L-Ala amidase), or Φ11 hydrolase (*N*-acetylmuramoyl-L-Ala amidase and D-Ala-Gly endopeptidase) releases surface protein with the predicted C-terminal cell wall anchor structures. In contrast to sortase A substrates, sortase B-anchored IsdC is attached to only six or seven disaccharide subunits and its wall peptides are either non-cross-linked (murein pentapeptides containing an extra C-terminal D-Ala) or linked to only two or three peptidoglycan subunits.

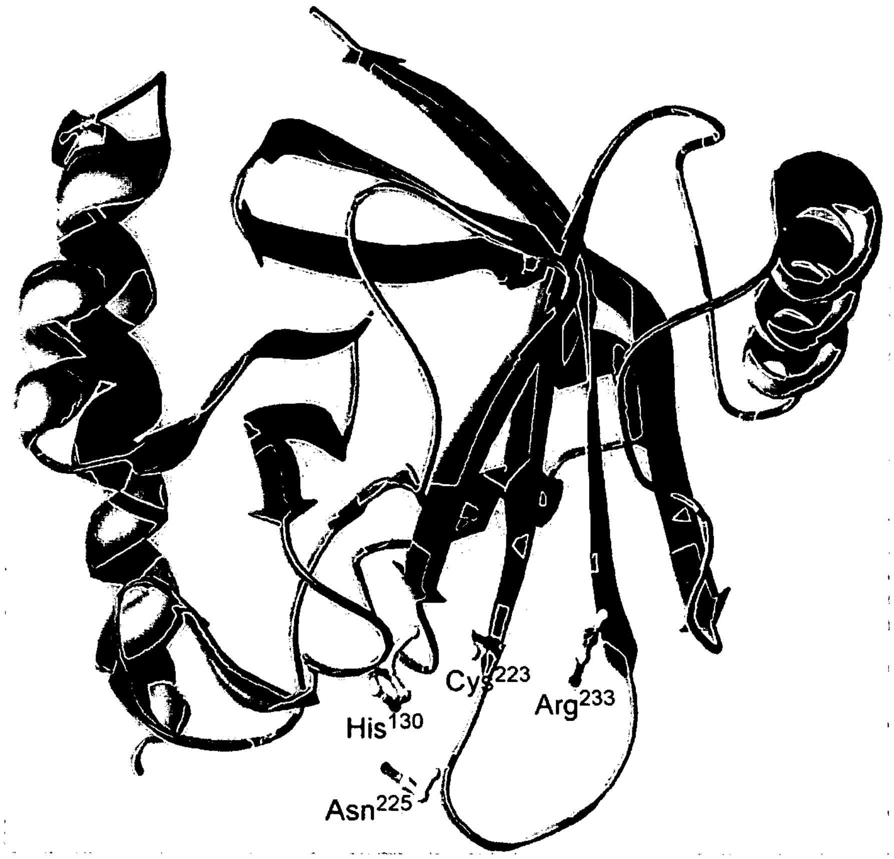

FIG. 8. Crystal structure of *S. aureus* sortase B. Sortase A and sortase B fold into very similar β-barrel structures; however, sortase B harbors three α helices that are absent in sortase A (here shown in orange) and that may contribute to the unique properties of sortase B substrate specificity and anchoring. Cys[223], His[130], and Arg[233] are equivalent to sortase A Cys[184], His[120], and Arg[197], respectively, and, along with Asn[225], presumably constitute the active site of sortase B (the structure was generated from atomic coordinates deposited in Protein Data Bank, PDB ID 1QXA) (228).

Sortase B Positions IsdC within the Cell Wall Envelope

The apparent lack of cross-linking of IsdC anchor peptides suggests that sortase B employs a unique cell wall substrate. Recent work proposed that the enzyme anchors IsdC to assembled (polymerized) peptidoglycan at a site with limited cross-linking (115). Considering that peptidoglycan strands may grow in a fashion that is perpendicular to the cell membrane (39) (as illustrated in Fig. 6B), attachment of protein to fully assembled peptidoglycan would enable a topology that encloses anchored protein in the cell wall envelope. It seems reasonable to presume that the enzyme properties in selecting peptidoglycan substrate are imprinted in the primary structures as well as the three-dimensional structures of sortase A and B. The polypeptide chain of sortase B is longer than that of sortase A. The two enzymes fold into very similar structures, i.e., β-barrels with short α helices connecting some of the β strands (225, 228) (Fig. 8). Two short α helices at the N terminus (α1 and α2) of sortase B, directly linked to the transmembrane segment of the enzyme, and a long α helix (α5) are absent in sortase A. It was proposed that α1 and α2 may project the enzyme active site towards the bacterial surface, whereas that of sortase A may face the plasma membrane (228). Is it really that simple? Turn the enzyme barrel 180° and, voila, the topology of anchored protein is changed? At this time, such a view is only speculation. Nevertheless, this hypothesis and others will guide future work aiming at the elucidation of the molecular mechanism whereby staphylococci position proteins at discrete locations in their cell wall envelope. As it is the case for sortase A, the two C-terminal β strands (β7 and β8) of sortase B form a groove where the active site resides. The structure in the presence of MTSET showed a disulfide bond between this inhibitor and the sulfhydryl group of Cys[223] (228), supporting the notion that this residue is the equivalent to sortase A Cys[184] (Fig. 8) (see above). The functional assignments of other residues within the sortase B active site are not at all clear. Zong et al. reported the presence of an arginine residue in the vicinity of Cys[223], i.e., Arg[233], the analog of Arg[197] in sortase A, and proposed that sortase B, or in general all sortases, generate a cysteine-arginine dyad, in which the sulfhydryl group is activated by the guanidinium group for catalysis (228). Supporting this hypothesis, the crystal structure of sortase B bound to MTSET and triglycine showed that the free amino group of this substrate was in close proximity to Arg[233] but far from His[130] (the sortase A His[120] analog) (228).

Zhang et al. suggested that the active site of sortase B may contain a cysteine-histidine-asparagine triad (Fig. 8, Cys^{223}-His^{130}-Asn^{225}) and proposed a catalytic mechanism that is similar to that of other cysteine proteases, in which the histidine imidazolium group assists the nucleophilic attack of the cysteine sulfhydryl group (225). Future advances towards understanding sortase B require improvements of the in vitro assay and experimental tests of structural predictions by analyzing single-residue substitutions.

SORTASE-CATALYZED POLYMERIZATION OF PILI

An additional and astonishing function of sortases is their involvement in the formation of pili in gram-positive bacteria. Pili, also known as fimbriae, represent proteinaceous filaments that protrude from microbial surfaces and typically function as a supramolecular structure, often with adhesive activity at their tip. The molecular mechanisms underlying the formation of pili in the outer membranes of gram-negative organisms are well understood (47). For gram-positive bacteria, electron microcopy experiments provided the first evidence for the presence of pili on the surfaces of *Actinomyces* spp., *Corynebacterium* spp., and *Streptococcus* spp. (27, 28, 102, 217, 218). As already reported for sortase-anchored surface proteins, the cell wall envelopes of gram-positive microbes appear to again serve as the assembly site for pili, which perform important functions during the pathogenesis of human or animal infections (203).

Actinomyces naeslundii

A. naeslundii is a human pathogen that can be isolated from the oral cavity and from supragingival dental plaque. Initial colonization of tooth or mucosal surfaces with *Actinomyces* spp. provides a biofilm substrate for the adherence of other plaque bacteria, including oral streptococci and several gram-negative bacterial species, which eventually leads to the formation of dental caries. Two types of fimbriae mediate adhesion of *Actinomyces* to tissue surfaces of the oral cavity. Type 1 fimbriae are required for binding tooth hydroxyapatite, whereas type 2 fimbriae mediate interaction with other bacteria and promote binding to the mucosal tissues of the host (203). Pioneering work carried out by Maria Yeung, John Cisar, and colleagues suggested an involvement of sortases in the formation of *A. naeslundii* fimbriae (222, 223). By generating *Actinomyces* plasmid expression libraries in *E. coli* and screening for plasmid clones that provided for immunoreactivity with antifimbrial serum, the genes encoding major pilin subunits of type I and type II fimbriae were isolated and named *fimP* and *fimA*, respectively (40, 219–221). DNA sequencing and analysis of genes surrounding *fimP* revealed its location within an operon containing seven open reading frames (ORFs): *orf3-orf2-orf1-fimP-orf4-orf5-orf6* (223). Of these, *orf1* and *fimP* encode a surface protein with LPXTG motif-type sorting signals, whereas the *orf4* protein product displays homology to sortase. Insertional mutagenesis of *orf4*, as well as *orf1*, *orf2*, *orf3*, or *fimP*, abolished bacterial adherence, suggesting that these genes are indeed required for the assembly of pili. Consistent with this hypothesis, FimP and Orf1 subunits accumulate in the envelope the *orf4* mutant strain, and fimbrial filaments are not assembled. Similar results were obtained for type 2 fimbriae after the analysis of the DNA sequences surrounding *fimA* (222), which is located in an operon containing three genes, *orf977-fimA-orf365*. While the first two genes encode surface proteins with LPXTG sorting signals, the product of *orf365* specifies a sortase homolog. Interruption of *orf365* abolished type II fimbrial assembly and led to the accumulation of FimA subunits with uncleaved sorting signals in the bacterial plasma membrane. These results suggest that *A. naeslundii* sortases are required for fimbrial assembly from precursor molecules that carry N-terminal signal peptides and C-terminal sorting signals.

Corynebacterium diphtheriae

C. diphtheriae is the causative agent of diphtheria, a deadly human disease that involves bacterial adherence to the upper respiratory tract and tissue damage via secretion of diphtheria toxin. The molecular mechanisms whereby *C. diphtheriae* binds to mucous membranes in the human pharynx and establishes a productive infection are still unknown. Following toxin-mediated tissue destruction and formation of pseudomembranes, associated inflammatory responses block human airways and precipitate respiratory failure (68). More than 30 years ago, Yanagawa and colleagues described pili on the surfaces of several different corynebacterial species, including *C. diphtheriae* (100, 217).

Bioinformatic analysis of the genome sequence of *C. diphtheriae* NCTC13129 identified six sortase-like genes (*srtA* to *-F*) (204). Five sortase genes are surrounded by ORFs encoding proteins with N-terminal signal peptides and C-terminal sorting signals, all clustered together in three separate loci on the bacterial chromosome. To analyze the expression and surface display of proteins, fragments of recombinant genes encoding signal peptides and sorting signals were expressed in *E. coli* and purified and antibody reagents were generated. Immunogold labeling of *C. diphtheriae* NCTC13129 followed by electron microscopy revealed that several of these antibodies stained pili on the bacterial surface. For example, antibodies raised against the SpaA (*s*ortase-mediated *p*ilin *a*ssembly A) protein stained filaments of 0.1 to 1 μm in length. SpaA protein is encoded by an operon comprised of four other open reading frames, *spaA-srtA-spaB-spaC*. Antibodies raised against purified SpaB also stained pili in immunogold labeling experiments, albeit that the gold particles were deposited at spaced intervals, whereas SpaA antibodies produced uniform staining. Antibodies raised against SpaC bound to the tip of the fiber and stained the same pili as SpaA- and SpaB-specific antibodies (Fig. 9).

Deletion of the *spaA* or the *srtA* gene completely abolished the assembly of SpaA pili as well as staining of pili with SpaB and SpaC antibodies. In contrast, deletion of *spaC* and *spaB* did not abrogate SpaA pilus formation. Transformation of pilin or *srtA* mutants with plasmid-carried wild-type genes restored the formation of pili and antibody staining (204). Pilus assembly can also be studied by immunoblotting. Pilin immune-reactive material must first be released from the bacterial envelope with muralytic enzyme and can then be loaded for sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). High-molecular-weight species that fail to separate on SDS-PAGE as well as monomeric pilin precursor

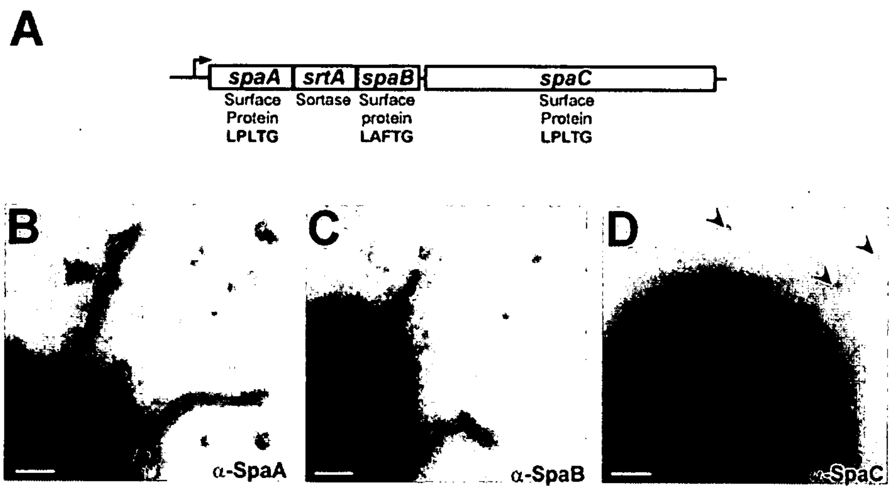

FIG. 9. *Corynebacterium diphtheriae* pili. A. Genetic organization of the *spa* locus of *C. diphtheriae* NCTC13129. Predicted promoters as well as the direction of transcription are shown with arrows. B to D. Corynebacterial pili stained with specific antiserum (anti-SpaA [B], anti-SpaB [C], or anti-SpaC [D]) and IgG-conjugated 12-nm gold particles. Samples were viewed by transmission electron microscopy. Bars indicate a distance of 0.2 μm. (Adapted from reference 204 with permission of Blackwell Publishing.)

molecules can be detected in this manner. When solubilized from extracts of *srtA* mutants, only SpaA pilin precursor, and not the assembled high-molecular-weight species, is detectable. These observations are consistent with the notion that SrtA catalyzes the assembly of SpaA pili and their deposition in the cell wall envelope. Polymerization of SpaA, SpaB, and SpaC into high-molecular-weight pili was examined in mutant strains lacking any one of the six different sortase genes. Only deletion of *srtA* prevented the incorporation of SpaA, SpaB, and SpaC into high-molecular-weight pili (204). SrtA alone appears to be sufficient for the polymerization of SpaA pili, as a variant with deletions of *srtB* to *-F* still produced high-molecular-weight immune-reactive material (199). As already observed by immunoelectron microscopy, deletion of *spaA* abrogated the incorporation of both SpaB and SpaC into pili when assayed by immunoblotting. In contrast, deletion of *spaB* or *spaC* did not abrogate the polymerization of SpaA (198, 204).

Taken together, these results suggest that *C. diphtheriae* pilus assembly is achieved by the activity of sortase A on pilin subunit precursors. As high-molecular-weight pili are resistant to boiling in SDS, it seems plausible that pilin subunits may be covalently cross-linked to one another. The requirement for sortase suggests that pilin subunit assembly involves not only the initiation of signal peptide-bearing precursors into the secretory pathway but also cleavage of sorting signals. SpaA, which represents the main pilin subunit, is uniformly present in the pilus shaft but apparently absent from the tip. Without SpaA, SpaB and SpaC cannot assemble into pili. SpaB, on the other hand, appears to decorate the shaft of SpaA pili, while SpaC may be positioned at the tip of this structure. Surprisingly, formation of this complex structure of SpaA pili requires only a single sortase gene, *srtA*. Whether this sortase can actually form transpeptidation products between proteins and, if so, what the nature of these linkages between pilus subunits is remain major research questions in this field. Interestingly, expression of the *A. naeslundii* pilin precursor FimA in *C. diphtheriae* led to formation of FimA pili (198). *C. diphtheriae* *srtD*, but not *srtA*, was required for the assembly of FimA pili, indicating that the mechanism of pilin polymerization is conserved among gram-positive bacteria.

How do sortases anchor some proteins to the cell wall envelope while assembling others into pili and still recognize precursor protein substrates at C-terminal sorting signals with strikingly similar properties? Pairwise comparison of the amino acid sequences of FimA, FimP, and SpaA identified four conserved sequence elements: (i) an N-terminal signal peptide, (ii) a C-terminal sorting signal, (iii) a central conserved domain with the amino acid sequence WxxxVxVYPK named the "pilin motif" (204), and (iv) a conserved domain with the amino acid sequence YxLxETxAPxGY, otherwise designated the "E box" (198). Alanine substitution experiments revealed that lysine 490 of the SpaA pilin motif is absolutely required for the polymerization of SpaA pili. As expected, mutations that perturb the LPLTG motif of the C-terminal sorting signal produced the same phenotype (204). Amino acid substitutions in the E box did not abrogate the polymerization of SpaA. Further, insertion of the pilin motif sequence into Seb flanked by an N-terminal signal peptide and a C-terminal sorting signal led to the polymerization of the reporter into high-molecular-weight polymers. Thus, the signal peptide, pilin motif, and sorting signal represent three topogenic elements that are necessary and sufficient for SpaA pilin polymerization by sortase.

The requirement for the single lysine residue within the pilin motif for assembly of pili is a compelling observation. Sortases require nucleophilic attack of amino groups at their acyl enzyme intermediates for product synthesis, i.e., a transpeptide bond between its two substrates. Therefore, it seems plausible that the ε-NH_2 group of the conserved lysine residue may perform a nucleophilic attack at sortase acyl enzymes charged with pilin substrate, thereby cross-linking two adjacent SpaA pilin subunits via a transpeptide bond (Fig. 10). SpaC does not harbor a pilin motif. The C-terminal sorting signal of SpaC permits formation of sortase acyl enzyme but cannot provide a pilin motif amino group for its resolution. It would follow that SpaC would be the first subunit

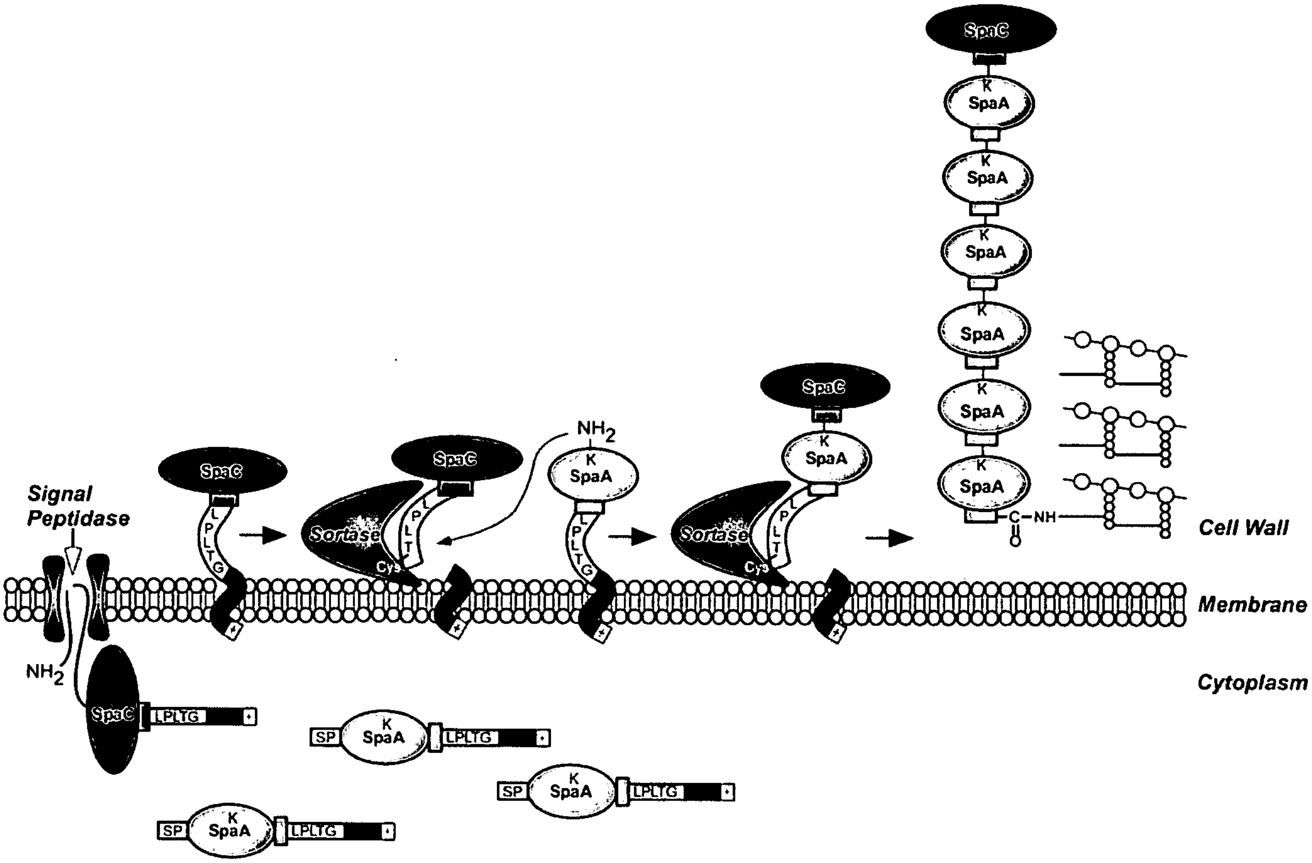

FIG. 10. Model for sortase-mediated pilus polymerization in *C. diphtheriae*. Sortase is thought to catalyze the polymerization of pili on the corynebacterial cell surface. Pilin subunits are typical sortase substrates, containing an N-terminal signal peptide (SP) that promotes secretion through the Sec system and a C-terminal cell wall sorting signal. SpaC is thought to be the first subunit to be incorporated into pili; if so, this might account for the detection of SpaC at the tip of the pilin fiber. The sortase-SpaC acyl intermediate may be attacked by the free amino group of a conserved lysine residue (K) present in the pilin motif of SpaA. The SpaA sorting signal would be in turn cleaved by sortase and linked to the lysine of a second SpaA pilin subunit. The remainder of the filament may then assemble by a sequence of similar transpeptidation reactions, and the polymerized pili may then be transferred to cell wall cross bridges for immobilization in the bacterial envelope. (Adapted from reference 203 with permission from Elsevier.)

incorporated into pili during the sortase-mediated pilin assembly pathway. Therefore, SpaC can be located only at the tip of the fiber that is assembled by a sequence of transpeptidation reactions. Sortase-mediated pilus assembly must occur in the vicinity of the plasma membrane, and the acylated enzyme may on occasion accept lipid II as a nucleophile, a mechanism that could provide for termination of assembly and for the anchoring of pili in the cell wall envelope. The incorporation of SpaB into SpaA pilin requires the conserved glutamic acid residue of the SpaA E box. However, alanine substitution of the conserved glutamic acid also interferes with SpaC incorporation into SpaA pili. Thus, the mechanism whereby SpaB is incorporated into SpaA pili remains unknown. Nevertheless, the intellectual framework provided by these studies has established a fertile testing ground for several different hypotheses that predict the polymerization of pili in several different gram-positive bacteria and the universality of the assembly mechanisms.

Streptococcus agalactiae

Identification of four topogenic sequence elements involved in the polymerization of pili on the surface of *C. diphtheriae* permitted bioinformatic analysis of other gram-positive genomes for the presence of pilus assembly genes. In addition to *A. naeslundii* and *C. diphtheriae*, *Bacillus cereus*, *Clostridium perfringens*, *Enterococcus faecalis*, *Streptococcus agalactiae*, and *Streptococcus pneumoniae* all carry pilin genes and associated sortases. Experimental verification of the elaboration of pili on the surfaces of these microbes is rapidly being garnered (102). Thus, why were these pili not detected in earlier studies that examined the morphology of gram-positive bacteria by electron microscopy? The answer seems surprisingly simple. The diameter of gram-positive pili is significantly less than that of their gram-negative counterparts, and the structures were often mistaken for artifacts (102).

Focusing on the development of a new vaccine for the prevention of group B streptococcal meningitis, researchers identified almost 600 open reading frames in the genomes of eight different *S. agalactiae* human meningitis isolates that encode secreted proteins or surface-associated factors (111). By purifying 312 recombinant proteins that are conserved among all eight isolates and testing these polypeptides in a newborn mouse model of group B streptococcal meningitis for protective immune responses, vaccine efficacy was assigned to a cocktail of four proteins: one secreted factor and three proteins bearing both an N-terminal signal peptide and a C-terminal sorting signal. Antibodies raised against two of the purified recombinant surface proteins stained *S. agalactiae* pili in immunogold electron microscopy experiments (102). Although the contribution of pilus fibers to the pathogenesis of *S. agalactiae* meningitis has not yet been established, it seems likely that the identified pili play important roles in bacterial attachment to host cells or invasion of specific tissues.

SORTASE AND SURFACE PROTEIN FUNCTION IN SELECT GRAM-POSITIVE BACTERIA

Listeria monocytogenes

L. monocytogenes, a food-borne, facultatively intracellular human pathogen, causes listeriosis. During the pathogenesis of human disease, listeriae cross intestinal, placental, or blood-brain barriers and, following invasion across the plasma membrane and escape from phagocytic vacuoles, replicate within epithelial cells or macrophages in an effort to escape innate immune responses (43, 208). Essential for this invasion strategy are two surface proteins named internalin A (InlA) and internalin B (InlB), which allow *Listeria* to target host cells by binding to specific surface receptors (33). Bacterial attachment to host cells triggers a signaling cascade that culminates in cytoskeletal rearrangements and phagocytic uptake of bacteria. The best-studied surface protein of *L. monocytogenes* is internalin A, a surface protein that mediates bacterial entry into intestinal epithelial cells (56). Internalin A binds E-cadherin receptors located primarily in the adherens junctions of epithelial cells (126). The N-terminal signal peptide of internalin A is followed by 15 leucine-rich repeats, which together are responsible for binding to E-cadherin receptor (103, 175), and a C-terminal sorting signal bearing an LPXTG motif. The genome of *Listeria monocytogenes* carries 43 genes with predicted sorting signals, the largest number of surface proteins encountered so far in a microbial genome (19, 63). Nineteen genes display homology with internalin A, indicating that an entire family of bacterial ligands for host cell receptors may be responsible for mediating listerial invasion of different host species or different cell types. Two sortase homologs have been identified in the sequenced *L. monocytogenes* genomes (63).

The cell wall anchor structure of internalin A in *L. monocytogenes* was elucidated. A methionyl–six-histidyl affinity tag was inserted just upstream from the LPXTG motif, and recombinant internalin A was expressed in *L. monocytogenes* EGD and purified after solubilization of the cell wall with endolysin (38). Phage-encoded endolysin functions as an endopeptidase and cleaves the L-Ala–D-isoGlu amide bond of listerial cell wall peptides (109). Mass spectrometry analysis of C-terminal internalin A anchor peptides indicated that the LPTTG motif is cleaved between the threonine and the glycine residues and that the C-terminal threonine forms an amide bond with the amino group of *m*-diaminopimelic acid, the cell wall cross bridge of listerial peptidoglycan (38). Thus far, this is the only surface protein anchor structure that was solved for a nonstaphylococcal protein. Nevertheless, the data indicate that sortase-mediated anchoring is a universal process recognizing shared features of polypeptide and peptidoglycan substrates.

As already mentioned, the *L. monocytogenes* genome sequence encodes two sortases. The 222-residue sortase A is 28% identical to *S. aureus* sortase A (11, 58). As expected, deletion of the *srtA* gene abolishes anchoring of internalin A to the cell wall (11). Immunoelectron microscopy as well as immunofluorescence microscopy showed that InlA is not displayed on the surface of *srtA* mutant *Listeria*. Immunoblotting of bacterial cell fractions indicated that internalin A is missorted to the medium, cytoplasm, and membrane in *srtA* mutant strains (11). The internalin sorting defect could be complemented in *trans* by introducing a plasmid encoding sortase A in mutant bacteria (11). Tandem mass spectrometry of peptides solubilized from purified peptidoglycan with trypsin indicated the absence of at least 13 LPXTG-containing surface proteins from the surface of *srtA Listeria*, with 6 of them absent from the nonpathogenic species *L. innocua* (11, 157). Interestingly, *L. monocytogenes* sortase A was able to anchor a fusion protein between internalin B, a protein otherwise targeted to the envelope by binding to lipoteichoic acid, and the *S. aureus* protein A sorting signal, revealing conservation of functional elements of the cell wall sorting pathway between these two bacterial species (11).

The role of sortase A in the anchoring of InlA and other internalins, which are known virulence factors, prompted an investigation of the effects of a *srtA* deletion on the pathogenesis of *L. monocytogenes*. The invasion properties of a *srtA* mutant were assessed in vitro in a gentamicin survival assay. Caco-2 epithelial cells and HepG-2 hepatocytes were infected with wild-type or mutant bacteria, and gentamicin was added to kill all noninternalized bacteria. The results revealed a severe defect in the internalization of *srtA* listeriae (11, 58), with values similar to those obtained for an *inlA* mutant (11). Interestingly, complementation of the *srtA* deletion with a single-copy insertion elsewhere in the chromosome (58), but not with the gene introduced with a high-copy plasmid (11), allowed the recovery of listerial invasiveness. This suggests that sortase A overexpression causes a dominant negative effect on the invasion of *L. monocytogenes*, probably by anchoring an excess of surface proteins that mask other surface factors required for invasion. In addition, wild-type and *srtA* bacteria were equally able to multiply inside macrophages, showing that the defect is specific to epithelial cells and hepatocytes (10). The contribution of sortase A to *L. monocytogenes* virulence was also examined following oral and intravenous infections. After oral inoculation of mice, *L. monocytogenes* is able to cross the intestinal barrier and colonize different organs in a manner that does not require internalin A. Quantification of bacteria in the liver and the spleen at 3 days postinfection indicated that the *srtA* mutant displayed, in comparison with the wild type, a 1- to 2-log-unit decrease in bacterial replication (11). As *inlA* mutants do not display a phenotype in this assay, it follows that other surface proteins must be important to establish listeriosis in this model. Intravenous injection of mice with wild-type *L. monocytogenes* is lethal in animals infected with a dose of 10^4 to 10^5 CFU. A similar level of mortality could be achieved by injecting 10^6 to 10^7 CFU of *srtA* bacteria, indicating an important defect in virulence for this strain (58). For example, when infected with 10^6 CFU of wild-type *L. monocytogenes*, mice succumb to infection within 4 days, whereas animals infected with *srtA* mutant bacteria survive this challenge. Quantification of *srtA L. monocytogenes* in spleen, liver, brain, and blood over a period of 7 days showed an increase in the bacterial counts during the first 4 days of infection, followed by a sharp decrease and bacterial clearance (58). The importance of *srtA* for listerial pathogenesis was corroborated in the guinea pig model of oral infection (167). After oral administration of *L. monocytogenes* EGDe or the *srtA* or *inlA* isogenic deletion mutant, the abilities of these strains to cross the intestinal barrier and colonize different organs were assessed. Bacterial counts for the *srtA* strain decreased by 3 log units in the intestine and 2 log units in the mesenteric lymph nodes compared to those for the wild type and by 1 log unit in both organs compared with those for the *inlA* strain. These experiments establish sortase A as a virulence factor of *L. monocytogenes* and suggest that in addition to InlA, other sortase substrates contribute to the observed defects of *srtA* mutants in listerial pathogenesis.

In an effort to identify such sortase A substrates, LPXTG-containing surface proteins present only in pathogenic *Listeria* species were analyzed to determine their role during infection. These studies revealed two new virulence factors: Vip (20) and InlJ (167). Vip, an LPKAG motif surface protein, does not belong to the internalin family but contains a proline-rich region. Immunofluorescence detection of this protein indicated that it is present in the bacterial cell wall, and this localization is dependent on the presence of sortase A but not sortase B (20). Using an *L. monocytogenes vip* mutant isogenic strain, it was determined that Vip is required for bacterial entry into Caco-2 and L2071 cells in vitro. In vivo, the contribution of Vip to listeriosis was assessed after oral infection of mice with wild-type, *vip* mutant, and *inlA* mutant strains. Quantification of bacteria in the intestine, lymph nodes, liver, and spleen indicated a reduction of several log units in the number of *vip* mutant bacteria compared with the wild type in all organs analyzed. As already mentioned, *srtA* but not *inlA* mutants display a similar phenotype; it follows that the absence of cell wall-anchored Vip is responsible, at least in part, for the *srtA* mutant virulence defect in this model. In addition, *vip* was shown to be required for listerial pathogenesis in the mouse model of intravenous injection and in the guinea pig model of oral inoculation (20). The analysis of proteins from Caco-2 and L2071 cell extracts with the ability to interact with Vip allowed the identification of the Vip ligand as Gp96, a protein present in eukaryotic cells and involved in the modulation of innate and immune responses (20). It is then hypothesized that Vip binding to Gp96 may impair its physiological function, thereby subverting the host immune response in a manner that facilitates listerial infections.

InlJ is one of the 19 InlA homologs present in *L. monocytogenes*, containing 13 leucine-rich repeat sequences (rich in cysteine) (167) and an LPKTG motif sorting signal. The isogenic *inlJ* mutant strain was impaired in its ability to colonize the liver and spleen after intravenous injection of mice compared to wild-type *L. monocytogenes* (167). However, the *inlJ* mutant showed no defect in the invasion of epithelial or endothelial cells, hepatocytes, or macrophages, challenging the classification of InlJ as an internalin. These results indicate that, while InlJ function remains elusive, this surface protein constitutes a novel virulence factor that contributes to the virulence defects of *L. monocytogenes srtA* mutants.

L. monocytogenes sortase B, a protein of 246 amino acids, is encoded by the *srtB* gene (11), which resides in an operon with an organization similar to that reported for staphylococcal *isd* (see above) (10, 181). The first gene of the operon, *lmo2186*, specifies an IsdC homolog containing an NPKSS motif (157) and is followed by *svpA*, encoding a polypeptide with weak homology to *S. aureus* IsdA and a sorting signal with a putative NAKTN motif. *lmo2184*, *lmo2183*, and *lmo2182* encode a putative lipoprotein, a membrane-anchored protein, and an ABC protein, similar to IsdD, IsdE, and IsdF of *S. aureus*, respectively. The *srtB* gene is positioned between *isdCDEF* and *isdG*, and the last gene of this locus encodes a protein with homology to the listerial phage protein Gp46. A Fur box is present in the promoter region of the listerial *isd* operon. The function of this element was assessed experimentally by placing *gfp* under the control of the *srtB* operon promoter (140). As expected, fluorescence of bacteria transformed with the *gfp* construct and grown in minimal medium was eliminated upon addition of $FeSO_4$. The notion that the listerial *isd* locus is expressed under iron-restrictive conditions is also supported by the observation that expression of SvpA is dramatically increased in media lacking iron and inside Caco-2 and HepG-2 cells.

The role of sortase B in listerial surface protein anchoring was investigated in a *srtB* mutant. Using polyclonal serum raised against proteins present in purified peptidoglycan, cell wall proteome expression was compared between wild-type, *srtA*, and *srtB* strains. While sortase A anchors the great majority of surface proteins to the cell wall envelope, sortase B is responsible for the surface localization of only a few polypeptides, among them SvpA (10). Immunofluorescence analysis supported this observation, as SvpA could be detected only on the surfaces of wild-type or *srtB*-complemented bacteria and not on the surfaces of *srtB* mutants. Interestingly, SvpA was detected either laterally along the bacterial cylinder or at one pole of *Listeria*, an observation that suggests some specialized type of anchoring by sortase B. Lmo2186, the *S. aureus* IsdC homolog, is also anchored by SrtB, as tandem mass spectrometry of peptides solubilized from purified peptidoglycan with trypsin indicated the absence of SvpA and Lmo2186 from the surface of *srtB Listeria* (157). Moreover, fusions of SvpA or Lmo2186 sorting signals to InlB were absent from the peptidoglycan fraction of *srtB Listeria*, a result that unequivocally defines these surface proteins as SrtB substrates.

Streptococcus pyogenes

S. pyogenes (group A streptococcus [GAS]) is a gram-positive extracellular human pathogen and is responsible for a wide spectrum of disease, ranging from localized suppurative infections such as pharyngitis to pyoderma to severe systemic illnesses (for example, pneumonia or septicemia) and to serious postinfection autoimmune sequelae exemplified by acute rheumatic fever and glomerulonephritis (34). Surface proteins play a major role in streptococcal virulence and have been studied for more than 50 years (50). Several surface proteins of *S. pyogenes* can be classified as LPXTG motif sortase substrates; among them are the adhesins protein M (75) and protein F (a fibronectin binding protein (177), the C5a peptidase (ScpA)

(25), and a protein G-related α2-macroglobulin-binding protein (159). Protein T, a trypsin-resistant surface protein, also harbors a C-terminal LPXTG motif sorting signal (172) and polymerizes into pilus structures that can be detected by immunoelectron microscopy (129). The antigenic properties of the M and T proteins are the basis for the serotype classification of GAS strains, and a combination of the recombinant versions of these proteins confer protection against mucosal challenge of mice with these pathogens (129).

A genetic screen was used to search for sortase genes, with the assumption that sortase-defective streptococci would fail to display protein F on the bacterial surface (7). Mutants generated by transposon mutagenesis were pooled and subjected to several rounds of immunoprecipitation with IgM, which precipitates bacteria that display protein F on their surface. Mutants unable to sediment upon incubation with IgM were mapped by DNA sequencing of insertion sites. One isolate harbored an insertion in the sortase A gene (*srtA*), and a deletion of this gene was generated by allelic replacement. The presence of several surface proteins in the bacterial envelopes of wild-type (M6 serotype) and *srtA* streptococci was detected by dot blotting using specific antibodies. Surprisingly, while the *srtA* mutant failed to display protein F, protein M, ScpA, and protein G-related α2-macroglobulin-binding protein on the bacterial surface, the anchoring and surface display of T protein were not affected by deletion of the *srtA* gene in spite of the presence of an LPXTG motif sorting signal in this polypeptide (7). However, deletion of a second sortase gene, named *srtB*, abolished the cell wall anchoring of T protein but had no effect on the anchoring and surface display of protein F, protein M, or ScpA. The *srtB* gene was found after bioinformatic searches of the *S. pyogenes* M1 genome for sortase homologs (7). The gene encodes a sortase with an N-terminal signal peptide and a C-terminal membrane anchor domain that is not structurally related to *S. aureus* SrtB. Additionally, in contrast to the case for *S. aureus* and *L. monocytogenes*, the *S. pyogenes srtB* gene is not associated with the *isd* locus but is present in an ~11-kilobase pathogenicity island known as the *f*ibronectin-binding, *c*ollagen-binding *T* antigen (FTC) region (9). While the genetic composition of the FTC island is highly variable, genes encoding T protein and SrtB homologs are always present in this region (129). As mentioned above, T proteins seem to be major subunits of *S. pyogenes* pili, whose assembly and surface display are dependent on sortase B (129). Together these results indicate that while *S. pyogenes* sortase A is able to anchor most LPXTG motif surface proteins, only sortase B can provide for the special linkage required for the polymerization of high-molecular-weight pili from T proteins.

S. pyogenes genomes harbor a variable number of four sortase genes (5, 48, 66, 132, 183, 189). Bioinformatic and Southern blot analyses of 12 different M serotypes showed that *srtA* is present in all strains, whereas *srtB* is present in only five of these isolates (7). Allele-specific PCR designed to detect all four sortases corroborated this finding (6). Analysis of 18 *S. pyogenes* isolates indicated that *srtA* is present in all strains examined, whereas *srtB* is present in fewer than half of all strains. Two other sortase genes (*srtC1* and *srtC2*) are only sometimes found in GAS strains. Interestingly, *srtC1* and *srtC2* have not yet been found together in streptococcal isolates. The *srtC1* and *srtC2* genes are flanked by ORFs that likely encode their surface protein substrates. In *S. pyogenes* MGAS315 (M3 serotype), the operon contains five ORFs: *cpa-sipA2-SPyM3_100-srtC2-SPyM3_102*, where *sipA2* encodes a putative signal peptidase and *cpa*, *SPyM3_100*, and *SPyM3_102* encode cell surface proteins with VPPTGL, QVPTGV, and LPLAGE sorting signal motifs, all of which diverge from the canonical LPXTG sequence. This finding triggered the question of which, if any, of these proteins were sortase C2-specific substrates. DNA sequences encompassing *sipA2-SPyM3_100* and *slpA2-SPyM3_100-srtC2* were cloned in an *S. pyogenes* plasmid and introduced into strain JRS4, a serotype M6 strain that lacks the *srtC2* locus (6). Detection of SPyM3_100 on the cell surface by dot blotting or in cell wall envelope fractions was shown to be dependent on the presence of *srtC2*. Replacement of the QVPTGV sequence with LPSTGE abrogated the anchoring of the mutant surface protein to the peptidoglycan. Thus, SrtC2 specifically recognizes and anchors proteins containing QVPTGV motif sorting signals. Interestingly, the amount of anchored product was significantly larger when *srtC2* and its substrates were expressed in a *srtA* strain than when they were expressed in wild-type streptococci. This observation suggests that sortases A and C2 may compete for the same cell wall substrate of the sorting reaction, presumably lipid II (see above).

Oral Streptococci

The production of acid and the ability to form biofilms with other microbes are attributes of *Streptococcus mutans* that aid in the development of human tooth decay (caries). Several cell wall-anchored surface proteins are involved in the attachment of bacteria to tooth surfaces or to other streptococci and actinomycetes that are present in mixed biofilms and in dental plaque (127). Surface protein P1 (also known as antigen I/II or Pac) is an adhesin that promotes bacterial colonization of tooth surfaces by binding to salivary agglutinin, a glycoprotein that coats teeth (127). P1 is a surface protein with an N-terminal signal peptide and a C-terminal LPXTG motif sorting signal. Cell wall anchoring and surface display of P1 require the sortase A gene (*srtA*) of *S. mutans* (81, 105). In contrast to *srtA* deletions in *S. aureus*, deletion of *srtA* caused mutant streptococci to secrete P1 into the culture medium (81, 105). The secreted species reacted with a polyclonal antibody raised against the sorting signal of P1 (105) and migrated more slowly than the species present in wild-type bacterial pellets (81), indicating that the uncleaved precursor is released into the medium by *srtA* mutants. Similar experiments demonstrated that *srtA* is required for the cell wall anchoring and surface display of glucan binding protein C (GbpC) (82) and dextranase (80), surface proteins with LPXTG motif sorting signals. GbpC promotes bacterial aggregation through binding of many different microbes to the same substrate molecule. As glucan is present on tooth surfaces, GbpC-mediated aggregation may contribute to plaque formation (127). Dextranase, on the other hand, is an enzyme that hydrolyzes the α-1,6 bonds of certain glucan molecules. This results in the alteration of the solubility and adhesive properties of the glucan substrate and promotes *S. mutans* biofilm formation (80). Other *S. mutans* LPXTG surface proteins are presumably anchored by sortase and involved in formation of oral cavities by this microbe, and these include fructosidase (18) and WapA (49, 158). As may be expected from the lack of anchoring of several adhesins, *S. mutans srtA* variants displayed a remarkable reduction in their ability to form biofilms (107), in the adhesion to saliva-coated hydroxyapatite in vitro and the colonization of rat teeth in vivo (105), and in the ability to aggregate in the presence of dextrose (82).

Interestingly, two *S. mutans* clinical isolates contain deleterious mutations in the *srtA* gene. *S. mutans* Ingbritt contains an 11-base-pair deletion in the *srtA* ORF that generates a premature stop codon (79). As a result, bacteria secrete P1, GbpC, and dextranase. Similarly, *S. mutans* NG5 carries a missense mutation in the *srtA* gene that results in the production of truncated, nonactive enzyme (106). This strain also secretes P1 and is unable to adhere to hydroxyapatite and to aggregate in the presence of saliva. These mutant phenotypes were reversed to the wild-type phenotype when sortase A from strain NG8 was expressed in *trans* (105).

Streptococcus gordonii, a commensal of the human oral cavity, also displays proteins on its surface that are essential for adhesion and colonization of the oral cavity. A combination of degenerate PCR and BLAST searches on the partially sequenced genome of *S. gordonii* allowed the identification and inactivation of the *srtA* gene in this bacterium (13). Mutant bacteria were unable to bind fibronectin, consistent with the lack of anchoring of several LPXTG motif surface proteins. More importantly, the ability to colonize the oral cavities of mice was significantly reduced in the *srtA* mutant compared with the wild-type strain.

Streptococcus pneumoniae: Surface Proteins and Pili

The host specificity of *S. pneumoniae* is mainly restricted to humans, where the organism colonizes the nasopharynx and respiratory tract and is the causative agent of otitis media, pneumonia, bacteremia, and meningitis (73). Like for many other gram-positive pathogens, several surface proteins with LPXTG motif sorting signals are known virulence factors of *S. pneumoniae*, including hyaluronidase (*hylA*) and neuraminidase (*nanA*), hydrolytic enzymes that degrade polysaccharides in the extracellular matrix and other bacteria (127). The two *S. pneumoniae* strains sequenced to date, R6 and TIGR4, each harbor a sortase A gene homolog, *srtA*. The *srtA* gene has been deleted using the R6 strain as a parent (92), and β-galactosidase surface display and NanA cell wall anchoring were abolished in the *srtA* mutant strain. The *srtA* mutant displayed defects in the ability of *S. pneumoniae* to attach and invade pharyngeal cells in vitro (92). A virulence defect could be detected in vivo in competitive infection studies using murine models for pneumonia, bacteremia, and nasopharyngeal colonization (150) and in the chinchilla model of nasopharyngeal colonization (26). These results suggest that *srtA* contributes to pneumococcal disease.

Signature-tagged mutagenesis experiments identified other sortase genes as virulence factors of the *S. pneumoniae* TIGR4 (serotype 4) encapsulated clinical isolate (71). Pooled insertional mutants, a total of 6,149 strains, were examined for the ability to cause lung infections in mice, and 387 insertional variants displayed an attenuated phenotype. Two of these strains contained transposon insertions in *rlrA* (for *R*ofA-*l*ike *r*egulator) and *srtD* (encoding a sortase homolog). In *S. pyogenes rofA* encodes a transcription factor that regulates the expression of protein F, a sortase A-anchored virulence factor (see above). Interestingly, the *rlrA* and *srtD* genes are located on a pathogenicity island, flanked by IS*1167* transposon elements (71) and present in a subset of clinical strains (150). *rlrA* is divergently transcribed from six other genes, three of which encode sortase homologs (*srtB*, *srtC*, and *srtD*) while the other three encode proteins with cell wall sorting signals. As expression of surface protein and sortase genes requires the RlrA transcription factor (72), the surface proteins were named RrgA, RrgB, and RrgC (*R*lrA-*r*egulated *g*ene). These proteins contain sorting signal motifs that diverge from the canonical LPXTG: YPRTG, IPQTG, and VPDTG, respectively. To examine the contribution of sortase and surface protein substrate genes to the pathogenesis of pneumococcal disease, all genes located in the pathogenicity island were mutated by in vitro transposition, and the virulence of the mutants obtained was assessed (71). Variants with transposon insertions in *rlrA*, *rrgA*, and *srtD* presented a virulence defect during murine lung infection. Moreover, when tested for colonization of the nasopharynx, *rrgA* and *srtB* variants were attenuated. Only the *rlrA* mutant displayed defects in the acute lethal disease following intraperitoneal injection of *S. pneumoniae*. It should be noted that the *rrgB* gene product encompasses not only an N-terminal signal peptide and C-terminal sorting signal but also a pilin motif and E-box sequence element. Although this has not yet been demonstrated experimentally, in accordance with the model for pilus assembly discussed above, the *rlrA*-regulated pathogenicity island of *S. pneumoniae* would be expected to provide for the expression of adhesive pili that aid in the pathogenesis of pneumococcal disease.

Streptococcus suis

S. suis, a chain-forming gram-positive pathogen, infects pigs and causes arthritis, meningitis, pneumonia, and endocarditis. *S. suis* is also known to cause meningitis in humans, and the microbe has been isolated from the respiratory and intestinal tracts of several ruminants. Two major *S. suis* virulence factors have been reported, a *m*uramidase-*r*eleased *p*rotein (Mrp) and an *e*xtracellular *f*actor (EF). The physiological and biochemical properties of these virulence factors are, however, still unknown (185). Mrp, a surface protein with a C-terminal sorting signal, is thought to be covalently anchored to the *S. suis* cell wall envelope, and this has stimulated the study of sortases in this microbe. As *S. suis* genomic sequences are not yet available, PCR amplification with degenerate primers was used to identify five sortase gene homologs, *srtA* to *-E*, in the genome of *S. suis* serotype 2, the most common disease-associated serotype (146). The *srtB* to *-D* genes are clustered with two genes encoding putative surface protein substrates, named *orf203* and *orf204*, specifying IPYTG and LPATG sorting signal motifs, respectively. *srtA* and *srtE* are located elsewhere on the chromosome. To characterize the role of these sortases in the anchoring of proteins to the cell wall of *S. suis*, three mutant strains lacking either *srtA*, *srtBCD*, or *srtE* were constructed. Purified *S. suis* cell wall sacculi from wild-type and mutant strains were treated with muramidase, and the solubilized proteins were separated by two-dimensional PAGE.

Compared with the cell wall proteome of wild-type streptococci, the envelope of the *srtA* strain lacked several polypeptides. In contrast, no differences in cell wall proteome were observed between the wild-type, *srtBCD* mutant, or *srtE* mutant strains. Edman degradation was used to identify Mrp and three other surface proteins with LPXTG motif sorting signals whose presence in the cell wall envelope required the *srtA* gene, consistent with the notion that sortase A anchors surface proteins with LPXTG motif sorting signals to the cell wall envelope. *S. suis* strains can be classified into 35 serotypes according to their polysaccharide capsular antigens, and this classification provides predictions of virulence and disease phenotypes (185). Not all serotypes appear to harbor the *srtA* gene, as Southern blot analysis failed to detect *srtA* in serotypes 20, 22, and 26 (145). It is not yet known whether the presence of *srtA* coupled with the ability to anchor surface proteins with LPXTG motif sorting signals exerts a general impact on the virulence of *S. suis* isolates.

Bacillus anthracis

B. anthracis is the causative agent of anthrax, a gram-positive spore-forming bacterium that predominantly infects herbivores (96). Humans and many different animal species are susceptible to *B. anthracis* infection. *B. anthracis* spores represent the infectious form of the pathogen and enter the host in three different ways, i.e., through a minor skin lesion, via inhalation, or via ingestion. Each of these entry routes leads to a different disease spectrum, commonly referred to as cutaneous, pulmonary, or gastrointestinal anthrax, respectively (128). The main virulence factors of *B. anthracis* include two secreted toxins, lethal toxin and edema toxin, as well as the cell wall-anchored poly-γ-D-glutamic acid capsule (22), which confers antiphagocytic properties on the vegetative form of bacilli. The genes encoding anthrax toxins or factors required for capsule production are located on two virulence plasmids, pXO1 and pXO2 (128).

The genome of *B. anthracis* carries three sortase genes (160). The predicted product of one of these genes, designated *srtA*, displays a high degree of amino acid sequence homology with *S. aureus* sortase A. The second gene is located within the *isd* locus of *B. anthracis*, which is predicted to be regulated by Fur and encodes surface proteins, an ABC transporter, a heme oxygenase, and a sortase B homolog (*srtB*). A third sortase gene, *srtC*, displays homology with *Bacillus* sp., *Streptomyces* sp., and *Clostridium* sp. sortases, enzymes that are found only in sporulating bacteria (41). Several genes encoding predicted surface proteins with LPXTG motif-type sorting signals were identified through bioinformatic searches of *B. anthracis* genome sequences. These surface proteins include many polypeptides of unknown function, several collagen binding proteins (216), and the receptor for the γ phage, a bacteriolytic phage specific for *B. anthracis* strains (35). To determine whether these polypeptides are anchored to the cell wall by sortase A, the *srtA* gene was deleted from the genome of *B. anthracis* strain Sterne, a variant lacking the pXO2 virulence plasmid, which provides for capsule biosynthesis (59). The cell wall anchoring of BasC (*Bacillus anthracis* surface protein *C*), a surface protein that functions as a collagen adhesin (216), was analyzed in wild-type as well as in *srtA* bacilli (59). Cleavage of the peptidoglycan strands with muramidase released FLAG epitope- and His_6-tagged BasC from the cell wall envelopes of bacilli. Recombinant BasC could be purified by affinity chromatography and detected by immunoblotting. BasC could not, however, be purified from the cell wall envelopes of *srtA* mutant bacilli, which harbored precursor polypeptide in membrane and cytoplasmic fractions. Cell wall anchoring of BasC in *srtA* mutant strains could be restored by expression of the *srtA* gene from a complementing plasmid. The contribution of *srtA* to virulence was assayed in the A/J mouse model of acute *B. anthracis* infection (59). A/J mice display a defect in the phagocytic killing of bacterial pathogens and, when infected with the attenuated *B. anthracis* strain Sterne, develop acute lethal disease symptoms. Mice were injected intravenously with wild-type and *srtA* spores. Time-to-death experiments indicated that the *srtA* mutant strain displayed no defect in the ability to cause an acute lethal infection in A/J mice. However, it was recently shown that *B. anthracis srtA* and *srtB* mutants are impaired in their ability to infect J774 macrophages (226), suggesting that, as is the case in the majority of gram-positive pathogens, *B. anthracis* sortases form part of the virulence repertoire of this microbe.

Recombinant *B. anthracis* sortase A was purified by affinity chromatography using His_6 replacement of the N-terminal signal peptide/membrane anchor. *B. anthracis* SrtA displayed a level of activity that is comparable to that of *S. aureus* sortase A, providing for an analysis of substrate specificity with FRET peptides (59). *B. anthracis* SrtA cleaved LPETG and LPATG fluorescent substrates, but not LPNTA, LGATG, or NPKTG; the latter peptide motif sequences are present in sorting signals of other *B. anthracis* surface proteins and presumably represent substrates for sortases B and C. Mass spectrometric analysis of *B. anthracis* SrtA cleavage products revealed that the enzyme cleaves LPETG substrate between the threonine and the glycine residue. This activity can be abolished with MTSET, and treatment with DTT restores enzyme activity. Thus, SrtA specifically cleaves LPXTG motif sorting signals and anchors surface proteins to the cell wall envelope of *B. anthracis*, similar to the case for sortase A of *S. aureus*. The biological functions of SrtB and SrtC and their protein substrates remain unknown. Future work is also needed to unravel the contribution of the three sortases to the pathogenesis of anthrax infections by using the fully virulent *B. anthracis* isolates as parent strains for mutagenesis and complementation studies.

Hyphal Development in *Streptomyces coelicolor*

Streptomyces coelicolor, a gram-positive filamentous bacterium in the soil, produces many natural antibiotics (74). The life cycle of this microbe begins with the formation of a feeding (submerged) mycelium, which differentiates into aerial hyphae that septate into spore chains. The hyphal surface becomes hydrophobic, a property that promotes outgrowth into the air and facilitates the dispersion of spores, which give rise to new mycelia. Six streptomycete surface proteins, collectively called chaplins, are involved in aerial hypha formation (29, 45). All chaplins are synthesized during mycelium formation as precursors with an N-terminal signal sequence and a short hydrophobic domain (chaplin domain) of about 40 residues. Chaplins D to H are small polypeptides, whereas chaplins A to C are larger and carry a C-terminal sorting signal (LAXTG motif). Deletion of six chaplin genes (*chpA* to *-H*) hindered the formation of aerial hyphae, and the defect could be rescued by the addition of purified chaplin proteins (45). As mixtures of ChpD to -H isolated from cell walls of aerial hyphae are highly surface active (29), it is presumed that these proteins would lower the water surface tension and thus allow the emergence of aerial hyphae at the air-water interface. In this model, the large chaplins ChpA to -C would be anchored by sortase to the bacterial peptidoglycan and would serve as a scaffold for the assembly of the small chaplins ChpD to -H. In addition, the chaplin layer provides a hydrophobic surface for the formation of the RdlA-RdlB rodlet layer, the highly insoluble, outer layer of proteins that facilitate spore dispersion (30).

BIOINFORMATIC ANALYSIS OF SORTASES AND SUBSTRATES

Through a variety of bioinformatic searches, sortase and sortase-like genes, along with a plethora of substrate genes, have been found in almost all gram-positive bacterial genomes available to date (12, 31, 41, 148). Additionally, sortase enzymes have been identified in the gram-negative organisms *Bradyrhizobium japonicum*, *Colwellia psychroerythraea*, *Microbulbifer degradans*, *Shewanella oneidensis*, and *Shewanella putrefasciens*, as well as in *Methanobacterium thermoautotrophicum*, a thermophilic archaeon (31, 147). Interestingly, in the majority of genomes where sortase enzyme genes have been identified, usually multiple sortases are encoded. Based on homology, the sortases thus far identified can be grouped into four or five subgroups or classes (Table 2). Each subgroup, in addition to distinctions in sequence, can be distinguished from one another based on membrane topology, genome position, and preference for substrates with specific amino acids within the cell wall sorting signal pentapeptide motif (31, 41).

The prototypical sortase A, first identified in *S. aureus*, contains an N-terminal transmembrane domain and the sequence TLXTC at its active site, where C corresponds to the catalytic cysteine residue (Cys^{184} in *S. aureus* sortase A [see above]). Sortase A appears to anchor a large number and broad range of surface proteins, and unlike many other sortase genes, the sortase A gene is not found clustered with its substrates. It also appears that only a single sortase A homolog is encoded per bacterial genome (31). The sortase A subgroup of enzymes also seems to share a preference for the LPXTG cell wall sorting signal motif. The second subgroup of enzymes, sortase B, along with its substrate (IsdC in *S. aureus*), is encoded in an iron transport operon involved in heme-iron uptake (see above) (41, 121, 182). Enzymes belonging to the sortase B subgroup contain three amino acid segments not found in sortase A and recognize substrates containing an NPQTN motif rather than the canonical LPXTG (41). The third class, designated sortase C or subfamily 3, contains a C-terminal hydrophobic domain (31, 41). This group of sortase enzymes is often encoded in multiple copies per genome. Subfamily 3 enzymes also share a preference for substrates containing the LPXTG cell wall sorting signal motif, often followed by a second G residue. Unlike those for sortase A, the genes for subgroup 3 enzymes are predicted to anchor a much smaller set of substrates, which are typically clustered with the structural gene for the enzyme (31).

A fourth subgroup can be defined after alignment of sortase sequences. It has been noted as the sortase D subgroup (41) or subfamilies 4 and 5, as sortases in this subgroup can be distinguished based on the cell wall sorting signals of their associated substrates (31). Sortases belonging to subfamily 4 are predicted to anchor proteins bearing the unique LPXTA(ST) motif (31). An alanine residue in the last position of the substrate motif suggests that the subfamily 4 enzymes fulfill a nonredundant role within the cell (31). These sortases are typically found clustered with their substrates, which usually possess enzymatic function. Many high-G+C bacteria contain sortases belonging to subfamily 5, and, interestingly, most do not harbor sortase A homologs. This subgroup of sortase enzymes shares substrate specificity for proteins containing an LAXTG motif (31), and at least in *Streptomyces coelicolor* they are essential for mycelium and hypha development (29, 45).

Many sortase genes are found clustered with genes encoding their substrates. *C. diphtheriae* serves as an example where sortases and their substrates cooperate to assemble pili. Five sortase genes are found in three loci on the corynebacterial chromosome, along with the various surface protein substrates that assemble into three types of pili (204). Genomic analysis also suggests that sortases and substrates belonging to different subgroups can be merged to form distinct sorting pathways (31). The *S. aureus isd* locus, for example, encompasses sortase B, its NPQTN motif substrate IsdC, and two other surface protein genes which are anchored by sortase A (IsdA and IsdB) (121, 182). Thus, while parallel pathways for sorting surface proteins to the cell wall envelope do exist, intersecting pathways appear to increase structural and functional flexibility. Once again, bacteria have evolved to make the most of their genes.

CONCLUSIONS AND FUTURE DIRECTIONS

Sortases are transpeptidases that cleave protein substrates at defined sites once they have been translocated across the bacterial plasma membrane. The resulting acyl intermediate between substrate and sortase is resolved by nucleophilic attack of an amine, typically provided by cell wall cross bridges but presumably also occurring for amino groups of proteins. The end product is the formation of a single amide bond with a surface protein substrate. Obviously, this bond would not be there if it were not for the ability of sortase to catalyze these reactions, without the need for high-energy phosphates. Is there more? Can sortases link proteins not just to cell wall and polypeptides? We do not know the answer yet, but shouldn't we simply ask why not? Amino groups exist on carbohydrates and lipids, and hence they could also be substrates for sortases.

Many surface protein substrates, sorting signals, and sortases have been identified, and the three dimensional structure of sortase with and without peptide substrate has been unraveled. In spite of this progress, we do not truly appreciate how these enzymes bind their peptidoglycan or protein cosubstrates, and we do not fully understand how their active-site thiol is activated for catalysis. Perhaps the most pressing need for our research is not to fill in the remaining puzzle of sortase reactions and enzyme-substrate relationships or to study their role in pathogenesis. Instead, since we already know that sortases are immensely important, we should concentrate our efforts on the discovery of small-molecule inhibitors that specifically block the sortase-catalyzed transpeptidation reaction, which, after all, does not exist in humans or animals. With a little bit of luck, such a therapeutic inhibitor may become a reality in the foreseeable future.

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Surface Proteins of Gram-Positive Bacteria and Mechanisms of Their Targeting to the Cell Wall Envelope

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INTRODUCTION

The cell wall of gram-positive bacteria is host to a wide variety of molecules and serves a multitude of functions, most of which are critical to the viability of the cell. Although the primary function of the cell wall is to provide a rigid exoskeleton for protection against both mechanical and osmotic lysis (694, 695) the cell wall of gram-positive bacteria also serves as an attachment site for proteins that interact with the bacterial environment. Over the past decade, it has become apparent that the gram-positive bacteria have evolved a number of unique mechanisms by which they can immobilize proteins on their surface. These mechanisms involve either the covalent attachment of protein to the peptidoglycan or the noncovalent binding of protein to either the peptidoglycan or secondary wall polymers such as teichoic acids.

This review describes our current knowledge about surface proteins of gram-positive bacteria and the mechanisms of their anchoring to the cell wall. Functions performed by the various wall proteins are incredibly diverse. For example, many covalently linked surface proteins of gram-positive pathogens are thought to be important for survival within an infected host (713). Other wall-targeted proteins are responsible for the controlled synthesis and turnover of the peptidoglycan at specific sites (division septa) during cell growth and division (348). It is believed that these enzymes are targeted to the division sites through a noncovalent interaction with specifically localized septal receptors. Still other surface proteins of gram-positive bacteria, including the internalin B molecule of *Listeria* spp., lysostaphin, and S-layer proteins, are immobilized to the cell surface by binding to secondary wall polymers present throughout the cell wall.

To facilitate this discussion of the mechanisms of protein attachment, we briefly discuss the mechanisms of protein secretion in these bacteria. We also summarize what is known about the structure, assembly, and turnover of the cell wall of gram-positive bacteria. For more detailed treatises on these subjects, we refer the reader to other excellent reviews (252, 707).

ARCHITECTURE OF GRAM-POSITIVE BACTERIA

Gram-positive bacteria are simple cells. On the basis of morphological criteria three distinct cellular compartments

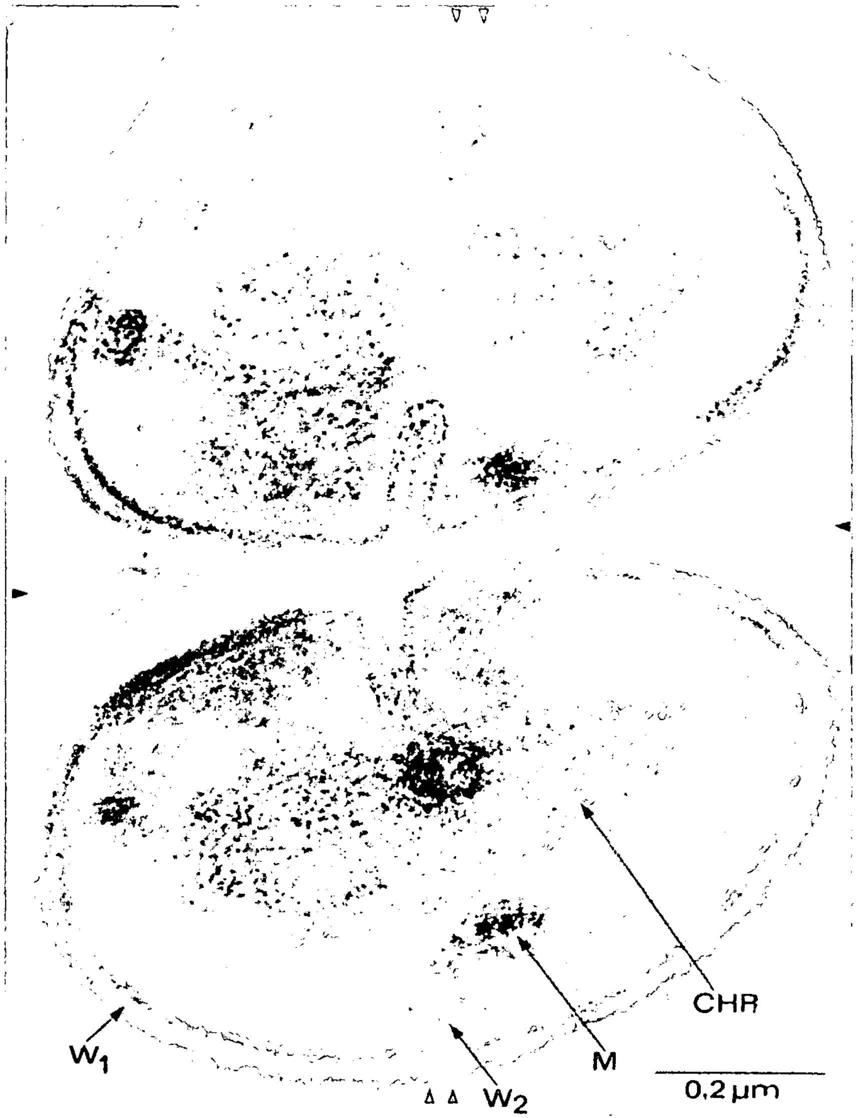


FIG. 1. Transmission electron micrograph of *S. aureus* cells undergoing cell division. Completed division septa are indicated by a single arrowhead, and new division sites are marked by double arrowheads. Newly formed cell wall (W_2) is distinguished from preexisting cell wall (W_1). Also visible are the bacterial chromosome (Chr) and cytoplasmic membranous bodies (M). This electron micrograph was generated by P. Giesbrecht. Reprinted from reference 260 with permission of the publisher.

can be distinguished: the cytosol, a single cytoplasmic membrane, and the surrounding cell wall (261). Some gram-positive bacteria synthesize a large polysaccharide capsule, whereas others elaborate a crystalline layer of surface proteins (739); both structures may envelope the entire cell. Spore-forming gram-positive bacteria, such as *Bacillus subtilis*, generate morphologically distinct daughter cells by a developmental program of asymmetric cell division (501). The cell wall of spores differs from that of mother cells and contains specific sets of proteins (645, 852). Some gram-positive bacteria divide without separating their cell walls and thus continue to grow as strings of cells (streptococci) or as clusters (staphylococci). Figure 1 is a transmission electron micrograph of thin-sectioned *Staphylococcus aureus* cells, revealing the characteristic morphology of the subcellular compartments of gram-positive bacteria.

SYNTHESIS AND STRUCTURE OF THE CELL WALL OF GRAM-POSITIVE BACTERIA

The cell wall of gram-positive bacteria is a peptidoglycan macromolecule with attached accessory molecules such as teichoic acids, teichuronic acids, polyphosphates, or carbohydrates (302, 694). The glycan strands of the cell wall consist of the repeating disaccharide *N*-acetylmuramic acid-(β1-4)-*N*-acetylglucosamine (MurNAc-GlcNAc) (254, 255). Glycan strands vary in length and are estimated to contain 5 to 30 subunits, depending on the bacterial species investigated (270, 331, 749). In most cases, the D-lactyl moiety of each MurNAc is amide linked to the short peptide component of peptidoglycan (256, 564, 792). Wall peptides are cross-linked with other peptides that are attached to a neighboring glycan strand (787–789, 791), thereby generating a three-dimensional molecular net-

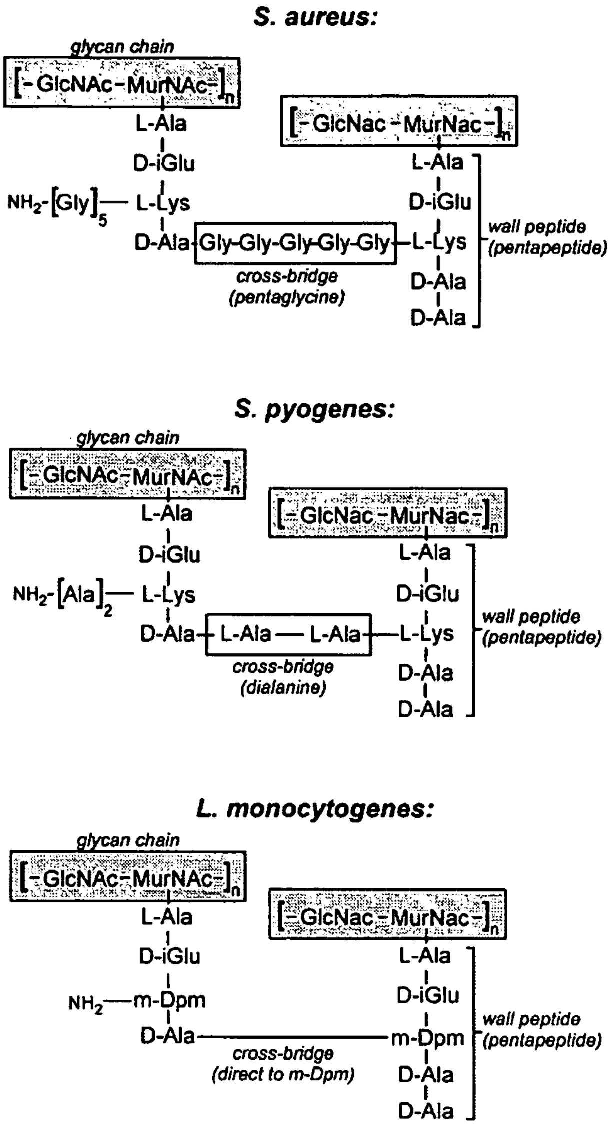


FIG. 2. Diagram of peptidoglycan structures from *S. aureus*, *S. pyogenes*, and *L. monocytogenes*. Glycan chains composed of a repeating disaccharide, GlcNAc and MurNAc, are linked to short wall peptides through the lactyl moeity of MurNAc. Adjacent wall peptides can be linked through crossbridge peptides (pentaglycine in *S. aureus* or dialanine in *S. pyogenes*). In some cases, such as *L. monocytogenes*, adjacent wall peptides are not cross-linked via peptide crossbridges. Instead, wall peptides are linked via an amide bond between the ε-amino group of a *meso*-diaminopimelic acid (m-Dpm) residue at position 3 of one wall peptide and the carboxy terminus of the D-alanyl residue at position 4 of the adjacent wall peptide.

work that surrounds the cell and provides the desired exoskeletal function (463, 760) (Fig. 2).

Cell wall synthesis in gram-positive bacteria can be divided into three separate stages that occur in distinct subcellular compartments, the cytoplasm, the membrane, and, finally, the cell wall itself (759, 761) (Fig. 3). Although we discuss here peptidoglycan synthesis in *S. aureus*, all bacteria use a nearly identical synthesis pathway. Peptidoglycan synthesis begins by generating UDP-MurNAc from UDP-GlcNAc and phosphoenolpyruvate (287, 758). Five amino acids are linked to UDP-MurNAc in four consecutive steps, L-Ala, D-isoGlu, L-Lys, and, finally, the D-Ala–D-Ala dipeptide (370, 554, 572). Synthesis of D-Ala–D-Ala requires two enzymes. The first, D-Ala racemase, converts L-Ala to D-Ala, while the second, D-Ala ligase, creates a peptide bond between two D-Ala residues (113, 578, 823). Synthesis of each peptide (amide) bond within the wall peptide consumes 1 high-energy phosphate (ATP) (812). The end product of the cytoplasmic segment of the cell wall synthesis pathway, UDP-MurNAc-L-Ala–D-isoGlu–L-Lys–D-Ala–D-Ala (UDP-MurNAc-pentapeptide, Park's nucleotide), has been solubilized by acid extraction of gram-positive cells and purified (616, 617). Radiolabeled Park's nucleotide was used as substrate for the in vitro synthesis of peptidoglycan, which provided a powerful assay for the elucidation of this biochemical pathway (116). UDP-MurNAc-pentapeptide is phosphodiester linked to an undecaprenyl-pyrophosphate carrier molecule at the expense of UDP (C_{55}–PP-MurNAc–L-Ala–D-isoGlu–L-Lys–D-Ala–D-Ala, or lipid I) (13, 371). UDP-GlcNAc is linked to the muramoyl moiety to generate the disaccharide lipid II precursor [C_{55}–PP-MurNAc(-L-Ala–D-isoGlu–L-Lys(Gly$_5$)–D-Ala–D-Ala)-β1-4-GlcNAc] (335–337). Lipid II is further modified by the addition of amino acids to the ε-amino of lysine (12, 417). Figure 3 shows these reactions for the biosynthesis of staphylococcal peptidoglycans, which contain five glycine residues linked to L-Lys. Three glycyl-tRNA species are thought to be dedicated to this biosynthetic pathway (281, 672, 673, 755). Finally, the modified lipid II precursor is translocated across the cytoplasmic membrane and serves as the substrate for the assembly of peptidoglycan (Fig. 3).

Cell wall assembly is catalyzed by penicillin binding proteins (PBPs) (251). The high-molecular-weight PBPs are bifunctional enzymes that have recently been categorized into one of two classes based on sequence similarity (251, 272). Class A PBPs promote both the polymerization of glycan from its disaccharide precursor, i.e., the successive addition of MurNAc (-L-Ala–D-isoGlu–L-Lys–D-Ala–D-Ala)-GlcNAc to C_{55}–PP-MurNAc(-L-Ala–D-isoGlu–L-Lys–D-Ala–D-Ala)-GlcNAc, and the transpeptidation (cross-linking) of wall peptides (570). The latter reaction results in the proteolytic removal of the D-Ala at the C-terminal end of the pentapeptide and the formation of a new amide bond between the amino group of the crossbridge and the carbonyl group of D-Ala at position 4 (791). The transpeptidation reaction of staphylococcal peptidoglycan is depicted in Fig. 3. This reaction is the target of penicillin and other β-lactam antibiotics which mimic the structure of D-alanyl–D-alanine (791). After cleavage, the β-lactam ring continues to occupy the active site serine residue of PBPs, thereby inhibiting PBPs (871, 872). Less is known about the class B PBPs; however, they are speculated to be involved in morphogenetic networks (272).

Not all cell wall peptides are cross-linked to their neighboring peptidoglycan strands. Unsubstituted (free) peptides may be preserved by trimming the terminal D-Ala off the pentapeptides (839). This reaction is catalyzed by other, low-molecular-weight PBPs that function as carboxypeptidases (372, 805). The reactions carried out by transpeptidases and carboxypeptidases are similar in nature. Transpeptidases use an amino group as a nucleophile to resolve the acyl-enzyme intermediate between the active-site hydroxyl of serine and the carbonyl of D-alanine, whereas carboxypeptidases use water as a nucleophile (251). Consequently, there are sequence and structural similarities between these enzymes (251). The ratio between transpeptidation and carboxypeptidation is thought to be important in generating a three-dimensional structure of the cell wall (463). For example, different degrees of cross-linking could allow wall synthesis at distinct angles and the generation of curves in otherwise cylindrical cells. However, this assump-

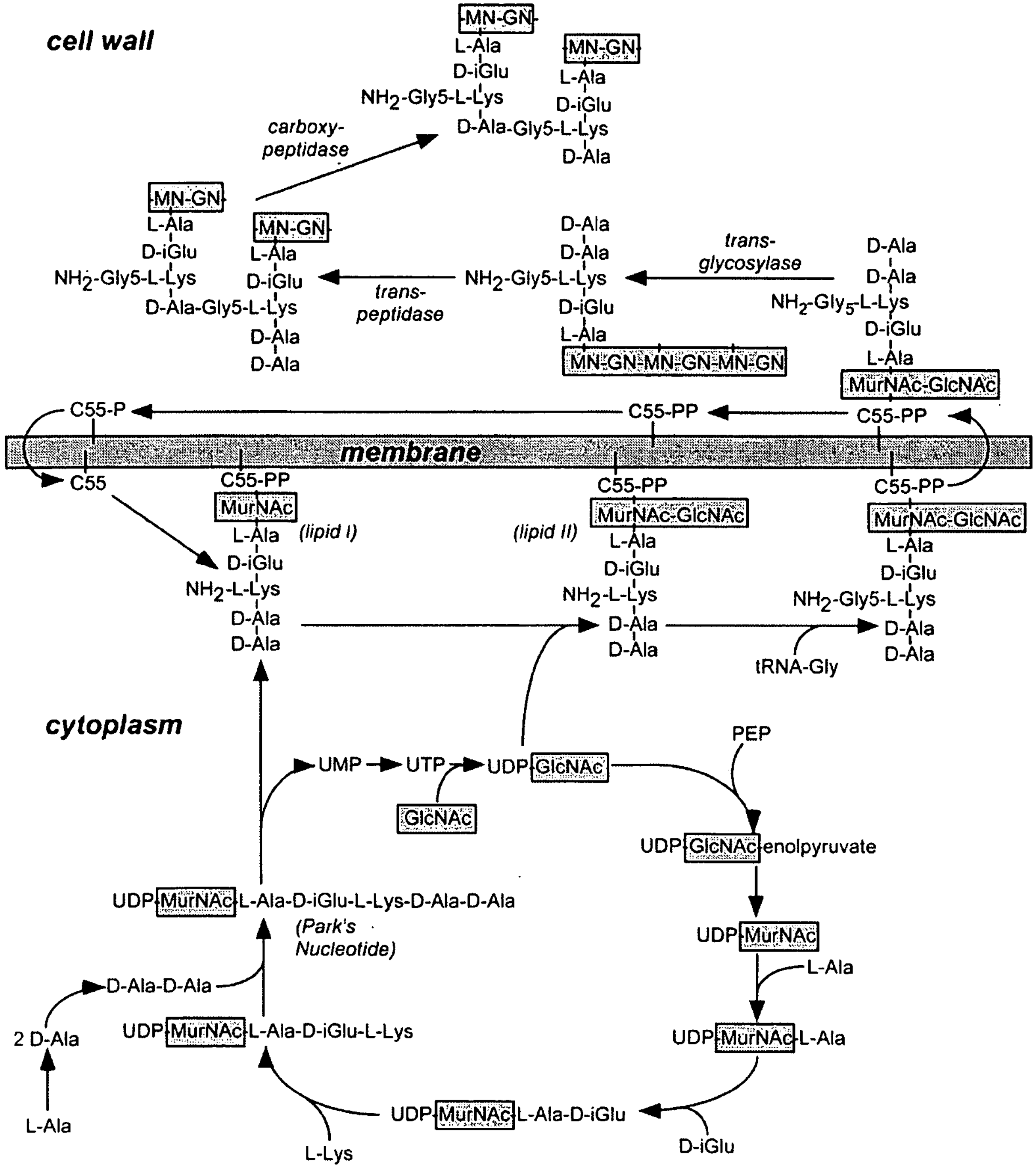


FIG. 3. Diagram of the biosynthetic pathway of cell wall assembly. As discussed in the text, the generation of cell wall precursors begins in the cytoplasm, resulting in the synthesis of UDP-MurNAc–L-Ala–D-iGlu–L-Lys–D-Ala–D-Ala (Park's nucleotide). The peptidoglycan precursor subunit is transferred to a lipid carrier in the membrane to generate lipid I. After further modification, the lipid-anchored peptidoglycan precursor is translocated to the extracellular face of the cytoplasmic membrane. The peptidoglycan precursor is subsequently incorporated into the cell wall by transpeptidation and transglycosylation reactions with the concomitant displacement of the lipid carrier. The terminal D-alanine of the wall pentapeptide is subject to substitution by cross-linking to other wall subunits or can be removed by the action of a D-alanyl–D-alanine carboxypeptidase. PEP, phosphoenolpyruvate; MN-GN, MurNAc-GlcNAc.

tion has never been tested for the distinctly shaped gram-positive bacteria. Peptidoglycan with a low degree of cross-linking is much more sensitive to degradation by cell wall hydrolases (760). It is conceivable that the degree of cross-linking plays a role in specifying sites on the cell wall that are more prone to either degradation or additional synthesis.

While the repeating disaccharide [MurNAc-(β1-4)-GlcNAc] is found in all bacterial peptidoglycans, the wall peptides differ in composition between bacterial species (710). Some organisms, such as *Listeria*, replace L-Lys with another diamino acid, in this case *m*-diaminopimelic acid (710). Others add amino acids to the side chain ε-amino of L-Lys to synthesize extended peptidoglycan cross-bridges (710). Figure 2 provides an example for the peptidoglycan structures of three different gram-positive bacteria. The wall peptides can either be cross-linked with those of another glycan strand such that the ε-amino of L-Lys or any other amino acid added at this position will be amide linked to the carbonyl of D-Ala in the wall peptide or be trimmed to generate an un-cross-linked wall tetrapeptide (710).

The cell wall of many gram-positive bacteria is modified by O-acetylation of MurNAc at C-6 (790). Although the enzymatic mechanism for this decoration remains unknown, it does confer resistance to cell wall degradation by animal lysozymes (97). Other chemical modifications include additions of teichoic acids, phosphorylation, and attachment of carbohydrates (see below). Recent advances in cell wall structure have been made by analyzing peptidoglycan breakdown products by reverse-phase high-pressure liquid chromatography combined with mass spectrometric analysis (144, 244, 269, 801). These techniques allow the characterization of different degrees of cross-linking as well as the identification of new cross-linking reactions within the cell wall (145).

CELL WALL-ASSOCIATED STRUCTURES OF GRAM-POSITIVE BACTERIA

Gram-positive bacteria synthesize several compounds that decorate their peptidoglycan exoskeleton (694). Based on structural differences, one can distinguish teichoic acids, teichuronic acids, lipoteichoic acids, lipoglycans, and polysaccharide modifications (694). Over the past 30 years, the complete structures of some of these compounds as well as the genes required for their synthesis have been characterized. This research has been reviewed in detail elsewhere, and we provide here a brief summary of these elements, since they may be important for protein targeting mechanisms (17, 205, 643).

Teichoic Acids

All gram-positive bacteria are thought to synthesize anionic polymers that are covalently attached to the peptidoglycan or tethered to a lipid anchor moiety (18, 23). Typically these polymers consists of polyglycerol phosphate (Gro-P), poly-ribitol phosphate (Rit-P), or poly-glucosyl phosphate (Glc-P) all of which may be glucosylated and/or amino acid esterified (205, 643). Figure 4 compares the structure of staphylococcal cell wall teichoic acid with that of two other bacteria. A polymer of 30 to 50 Rit-P subunits is phosphodiester linked to 2 or 3 Gro-P residues which are linked to the disaccharide Man Nac(β1-4)GlcNAc (16, 183, 184, 700, 701). The GlcNAc moiety is (1–6) phosphodiester linked to MurNAc within the peptidoglycan repeating disaccharide (MurNAc-GluNac) (128, 318, 438). The $(\text{Gro-P})_n$-ManNac-GlcNAc tether between wall teichoic acids and the peptidoglycan is referred to as the linkage unit (15). Although many gram-positive bacteria are known to synthesize identical linkage units, some species modify its structure with other carbohydrates whereas a few others use entirely different compounds to tether their wall teichoic acids (15).

Modifications of the repeating subunits differ from one bacterial species to another (17). Both Gro-P and Rit-P repeating units can be decorated with a variety of different sugars and can also be esterified with D-alanine (78–80). Synthesis of the backbone structure is thought to occur in the bacterial cytoplasm (78, 643). Synthesis begins by fusing UDP-GlcNAc to prenol-phosphate (862, 863) (Fig. 5). The resulting GlcNAc-PP-prenol is then mannosylated with UDP-ManNac to yield ManNac-GlcNAc-PP-prenol (873). The lipid-anchored cell wall linkage unit serves as an attachment site for the addition of two or three Gro-P units and is extended by Rib-P, which is added from CDP-ribitol substrates (267, 268, 521, 642). The end product of this synthetic pathway is presumably translocated across the cytoplasmic membrane via an ATP binding cassette transporter system (472). Attachment of wall teichoic acid to MurNAc proceeds during cell wall assembly; however, the enzymatic machinery required for this process is still unknown.

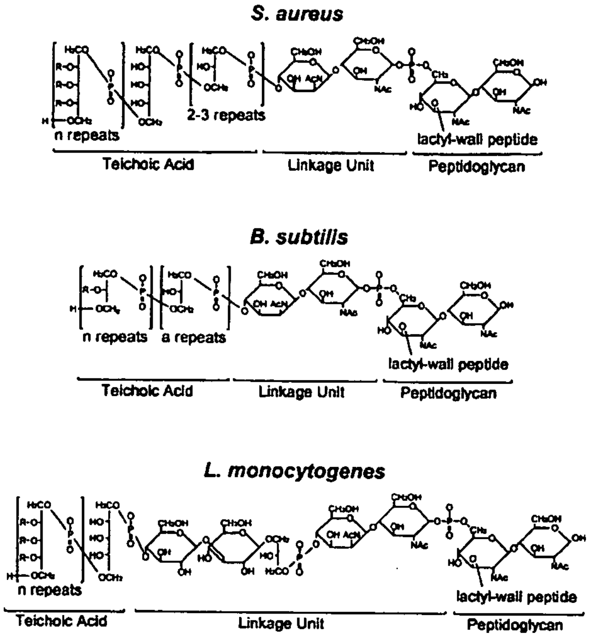


FIG. 4. Structure of cell wall teichoic acids from *S. aureus*, *B. subtilis*, and *L. monocytogenes*. Wall teichoic acids are anionic polymers of glycerol-phosphate, ribitol-phosphate, or glucosyl-phosphate and are tethered to the peptidoglycan. The cell wall linkage unit consists of ManNac(β1-4)GlcNAc, which is 1–6 1-6phosphodiester linked to MurNAc within the glycan strands of the cell wall envelope. One to three glycerol-phosphate moieties serve as a bridge between the linkage unit and the anionic polymer, which is composed of 15 to 60 subunits. R indicates covalent modification of the hydroxyl side chains of either glycerol-phosphate or ribitol-phosphate and can be either GlcNAc or esterified D-alanine.

Esterification of Gro-P or Rit-P with D-Ala occurs on the surface of the cytoplasmic membrane, i.e., after teichoic acids have been translocated across the membrane (580). Four gene products are required for this process, during which D-Ala is first linked to a diacyl carrier protein and then linked to an undecaprenol phosphate lipid carrier (139, 316, 317, 580, 592, 641). Lipid-linked D-Ala is translocated across the cytoplasmic membrane and serves as a substrate for esterification. This reaction is presumably used for the D-Ala ester modification of cell wall teichoic acids and lipoteichoic acids (579). Cell wall teichoic acids are thought to be uniformly distributed over the entire peptidoglycan exoskeleton (806). Some teichoic acid decorations, for example the esterified D-Ala, are unstable, and *S. aureus* continuously reesterifies D-Ala residues (431, 433).

Although the structures of cell wall teichoic acids are largely known and some of the genes involved in their synthesis appear to be essential for the growth of gram-positive bacteria, the physiological role of these molecules is still not completely understood (642). It is conceivable that the negatively charged teichoic acids function to capture divalent cations or provide a biophysical barrier to prevent the diffusion of substances (205,

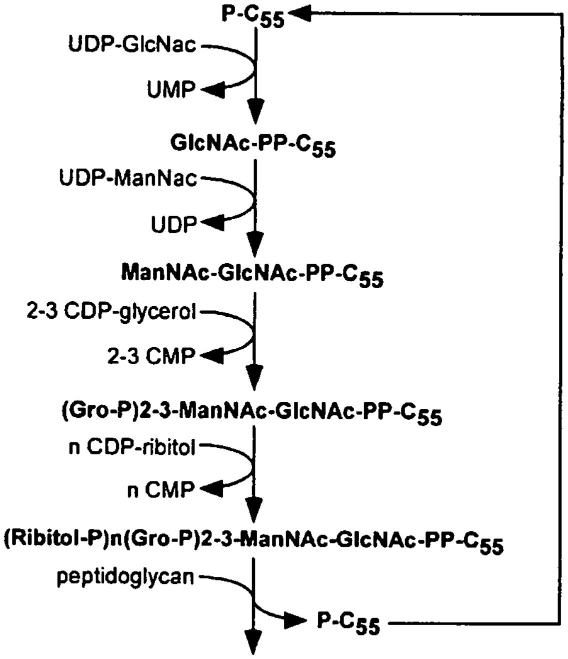

P-C55
UDP-GlcNac
UMP
GlcNAc-PP-C55
UDP-ManNac
UDP
ManNAc-GlcNAc-PP-C55
2-3 CDP-glycerol
2-3 CMP
(Gro-P)2-3-ManNAc-GlcNAc-PP-C55
n CDP-ribitol
n CMP
(Ribitol-P)n(Gro-P)2-3-ManNAc-GlcNAc-PP-C55
peptidoglycan
P-C55
(Ribitol-P)n(Gro-P)2-3-ManNAc-GlcNAc-P-peptidoglycan

FIG. 5. Diagram of the biosynthesis of staphylococcal cell wall teichoic acid. Synthesis begins in the cytoplasm via the addition of UDP-GlcNAc to an undecaprenol carrier molecule (P-C_{55}). UDP-ManNac and 2,3-CDP-glycerol are added to complete the cell wall linkage unit, which is then extended by the polymerization of ribitol-phosphate. This precursor molecule is thought to be translocated across the cytoplasmic membrane and linked to MurNAc within the glycan chains.

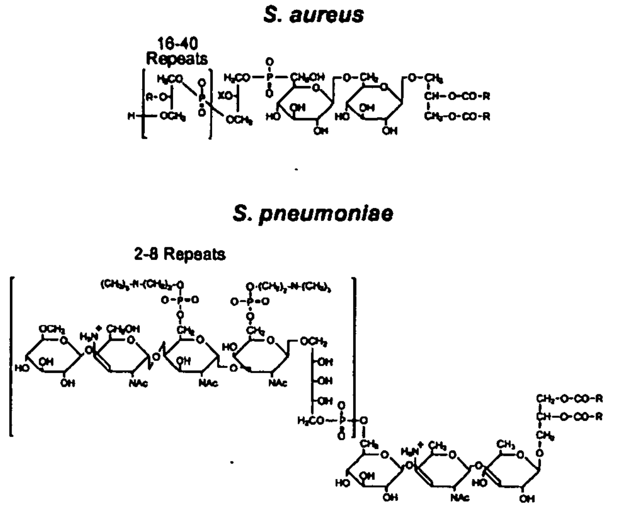


FIG. 6. Structure of lipoteichoic acid from *S. aureus* and *S. pneumoniae*. Staphylococcal lipoteichoic acid is composed of polymerized glycerol-phosphate and is linked to a membrane anchor moiety [Glc(β1–4)Glc(β1–3)diacylglycerol]. The fatty acid side chains of diacylglycerol are indicated by R. The hydroxyl side chains of polymerized glycerolphosphate are modified with GlcNAc or esterified D-alanine. In *S. pneumoniae*, the repeat unit of lipoteichoic acid, [Glc(β1–3)AATGal(α14)GalNac(6-Cho-P)(α1–3)GalNAc(6-Cho-P)(β1-1)ribitol-P], is identical to that of cell wall teichoic acids. The choline-modified repeat unit serves as a targeting receptor for several murein hydrolases and surface protein. Modified from reference 205 with permission of the publisher.

207). However, these claims have been largely speculative and experiments that directly prove or disprove them are difficult to design. Cell wall teichoic acids appear to be the binding sites for some enzymes that cleave the bacterial peptidoglycan (333). For example, the LytA amidase of *S. pneumoniae* binds to the choline moiety of the cell wall teichoic acids of this organism (351, 352). Conceivably, the affinity for teichoic acids directs murein hydrolases to the cell walls of specific species (discussed below). Thus, teichoic acids may serve as species-specific decorations which allow gram-positive bacteria to synthesize an envelope structure that is chemically distinct from the envelope other organisms that display an otherwise identical peptidoglycan exoskeleton.

Lipoteichoic Acids

Lipoteichoic acids are polyanionic polymers inserted in the outer leaflet of the cytoplasmic membrane via a lipid moiety (205). The polymer extends through the cell wall peptidoglycan onto the surface of gram-positive cells. The precise function of lipoteichoic acids is unknown (205). The cell wall teichoic acids and lipoteichoic acids possess different chemical structures in all gram-positive bacteria except *Streptococcus pneumoniae*. For example, *S. aureus* synthesizes poly-Rit-P cell wall teichoic acids, which are modified at the 2′ and 4′ hydroxyl with GlcNAc and D-Ala (701). Staphylococcal lipoteichoic acids are composed of poly-Gro-P, which can be modified at the 2′ hydroxyl of Gro-P with either GlcNAc or esterified D-Ala (165, 208–210). The lipoteichoic acid moiety of *S. aureus* is attached to Glc(1-4)Glc-(1-3)diacylglycerol (433).

Lipoteichoic acids of other bacteria have different repeating subunits and/or different lipid anchors. Figure 6 compares the structure of staphylococcal lipoteichoic acid with that from *S. pneumoniae* (205). Lipoglycans are related structures that can be distinguished from lipoteichoic acids by the absence of phosphate from the repeating subunits (205). Synthesis of the lipoteichoic acids is thought to occur on the surface of the cytoplasmic membrane. Thus, the substrates of each lipoteichoic acid constituent must be translocated across the cytoplasmic membrane prior to assembly (205). Indeed, each of the precursor molecules is tethered to a lipophilic moiety: either glycolipid, phosphatidylglycerol, hexosyl-1-phosphoundecaprenol, or undecaprenyl-D-Ala (106, 241) (Fig. 7).

Lipoteichoic acid synthesis begins in the cytoplasm with the synthesis of phosphatidic acid from glycerol, ATP, and two acyl side chains (Fig. 7). Phosphatidic acid is converted to phosphatidyl-glycerophosphate and then to phosphatidylglycerol, which serves as a substrate for the polymerization of Gro-P (266). This synthetic scheme requires large amounts of diacylglycerol, which are recycled to phospatidic acid or used for the biosynthesis of other membrane lipids (433). Glycosylation of lipoteichoic acid requires hexose linked to undecaprenyl, which is presumably synthesized in the cytoplasm and translocated across the membrane (206, 432, 482). It is conceivable that lipoteichoic acids, similarly to the cell wall teichoic acids of *S. pneumoniae*, also serve as species-specific decorations of the peptidoglycan exoskeleton.

PROTEIN SECRETION IN GRAM-POSITIVE BACTERIA

Protein secretion has been studied extensively in *Escherichia coli*, and the paradigms established through this work are thought to be true for all bacterial and eukaryotic cells (171, 640, 654). Proteins destined for translocation across the cytoplasmic membrane are marked by a signal (leader) peptide (65) that is generally composed of a core of 15 to 20 hydrophobic residues flanked at the N-terminal end by positively

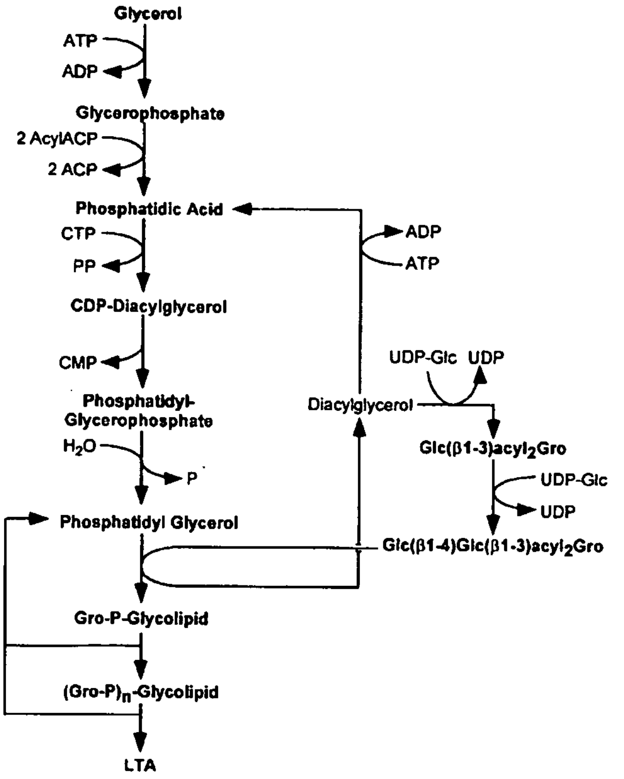


FIG. 7. Diagram of the biosynthesis of staphylococcal lipoteichoic acid (LTA). Glycerol-phosphate is acylated with two fatty acids to yield phosphatidic acid. This compound, as well as all other biosynthetic components of lipoteichoic acid, are thought to be located in the outer leaflet of the cytoplasmic membrane, the presumed site of lipoteichoic acid synthesis. The glycolipid anchor, Glc (β1–4)Glc(β1–3)diacylglycerol, is linked to the first glycerol-phosphate moiety and then further extended by polymerization with phosphatidyl-glycerol as a substrate. Modified from reference 205 with permission of the publisher.

charged residues (182, 728). For secreted proteins, signal peptides are proteolytically removed by signal (leader) peptidases upon translocation across the cytoplasmic membrane (136, 153). Signal peptides are necessary and sufficient for protein translocation across membranes if the fused polypeptide substrate can be maintained in an export competent state, a function that can be achieved by one of two separate pathways (142, 808). In one pathway, a signal recognition particle (SRP) can bind to the signal peptides of nascent chains and temporally arrest their ribosomal translation (824–826). The SRP of *E. coli* is thought to be a ribonucleoprotein particle consisting of Ffh (also known as P48) and 4.5S RNA (46, 648, 678, 762). Translation resumes after the SRP-ribosome complex docks onto its membrane receptor (FtsY), thereby delivering the nascent polypeptide to the Sec translocation channel (551, 808). Alternatively, signal peptide-bearing precursors may be translocated after their synthesis has been completed, i.e., by a posttranslational translocation process. Binding of a secretion chaperone, SecB, can maintain these precursors in an unfolded, translocation-competent state (452, 661). The SecB protein binds to the mature part of signal peptide-bearing precursors but may also interact with the SecA protein, a component of the secretion machinery itself (90, 151, 341, 662). Once initiated into the secretory pathway by the signal peptide, the SecB chaperone dissociates from the precursor, allowing the translocation of the full-length polypeptide across the membrane (197). The pathways converge at the translocation channel, and the choice of pathway that is used may be a function of the hydrophobicity of the signal peptide (807, 808). Recent observations suggest that the SRP pathway may target proteins destined for the inner membrane to the Sec translocase whereas the SecB pathway seems to be used by proteins that are secreted across the membrane (721, 804).

The gene products necessary for the initiation of leader peptide-containing precursors into the secretory pathway were identified as mutants that restored the β-galactosidase activity of LacZ fusions to the C terminus of signal peptide bearing proteins (600). These LacZ fusion proteins are substrates for export but arrest at an undefined step of the secretion pathway and block the export of other precursors. The results of the *sec* screens generally matched those of a suppressor screen (*prl*, for "protein localization") that scored for the export of a mutant polypeptide harboring a defective signal peptide (181). The functionality of the Sec proteins in membrane translocation of precursor proteins has been elegantly demonstrated in vitro (309, 563). SecYEG is the preprotein translocase channel and requires the SecA ATPase to push polypeptides through a hydrophilic channel (172, 174, 179, 811). The SecDF and YajC proteins function at a later step, perhaps in regulating the activity of SecA (173).

Homologs of SecA, SecD, SecE, SecF, SecY, and YajC have been identified in the sequenced genome of the gram-positive organism *B. subtilis*; however, no homologs of SecB and SecG were found (454). It is not clear whether SecB and SecG are dispensable for secretion in *B. subtilis* or whether they have been replaced with other polypeptides that do not display homology to the *E. coli* gene products. Ffh, 4.5S RNA, and FtsY are conserved between *E. coli* and *B. subtilis* (102, 456, 774). Another striking difference between *E. coli* and *B. subtilis* is the number of signal peptidases. The gram-positive organism appears to encode seven signal peptidases, whereas *E. coli* contains only two. Two of the *Bacillus* genes specify class 2 signal peptidases for the maturation of lipoproteins, whereas the other five signal peptidases are similar to the *E. coli lepB* gene. Signal peptides of gram-positive bacteria differ from those of their gram-negative counterparts by usually being longer and more hydrophobic and possessing more charge in their N-terminal ends (818, 819). Although these signal peptides of gram-positive organisms generally function in gram-negative bacteria, the same is not always true when signal peptides of gram-negative bacteria are expressed in gram-positive organisms (716). Could it be that gram-positive bacteria require additional components to recognize signal peptides? This has been tested biochemically for both *B. subtilis* and *S. aureus* by purifying membranes with bound ribosomes and comparing their protein content with the content of membranes those that did not contain ribosomes (2, 3). Although several different polypeptides could be identified as a secretory (S) complex, analysis of the cloned sequences revealed pyruvate dehydrogenase, an enzyme of intermediary metabolism that is presumably not directly involved in protein secretion (329, 330).

A "PERIPLASMIC SPACE" IN GRAM-POSITIVE BACTERIA?

The existence of a periplasmic space in gram-positive bacteria that is analogous to the one found in gram-negative bacteria has been a matter of speculation for many years (276). Morphological inspection has at times revealed a narrow space between the cytoplasmic membrane and the cell wall peptidoglycan (276). Visualization of this space depended on the mode of sample preparation (275). Others have emphasized an operational definition for the periplasm of gram-positive bacteria as a subcellular compartment (544); i.e., it should contain a subset of secreted proteins that are specifically targeted to this compartment (644).

Several proteins that are periplasmic in gram-negative bacteria were observed to be lipid modified in gram-positive bacteria (264). These lipoproteins function to capture specific import substrates, such as carbohydrates, and deliver them to transport machinery embedded within the cytoplasmic membrane. The homologous proteins in gram-negative cells are generally soluble in a periplasmic space that is bounded by both the bacterial inner and outer membranes. Hence, the lipoyl moiety might be a targeting device to retain polypeptides on the membrane surface of gram-positive bacteria (264, 769, 770). For example, β-lactamase (Bla) is a soluble periplasmic enzyme that confers resistance to β-lactam antibiotics in gram-negative bacteria (444) while the β-lactamases of *S. aureus* and *B. licheniformis* are lipoproteins (584, 585). β-Lactamase precursors in gram-positive bacteria appear to be substrates for both type I and type II signal peptidase, resulting in mixed populations of secreted, soluble Bla as well as glyceride-modified, membrane-anchored Bla (573). Mutant Bla exported solely by a type I signal peptide is secreted into the extracellular medium but fails to protect staphylococci from β-lactam antibiotics (573). Thus, tethering of such enzymes to the cytoplasmic membranes is an important factor in both lowering the local concentration of inhibitors such as penicillin and raising the local concentration of necessary nutritional resources.

HISTORY OF SURFACE PROTEIN ANCHORING IN GRAM-POSITIVE BACTERIA

The high incidence of streptococcal and staphylococcal human diseases at the beginning of this century sparked the interest of many medical investigators in these microbes. Initial efforts concentrated on the characterization of the predominant antigens during infection by gram-positive bacteria with the rationale of developing protective vaccines (285, 466). Although vaccines for *Streptococcus pyogenes* and *Staphylococcus aureus* are still not commercially available, this research rapidly identified and characterized protein and carbohydrate structures on bacterial surfaces. Lancefield and colleagues classified streptococci on the basis of carbohydrate antigens. The group A streptococcus (GAS), *S. pyogenes*, is the causative agent of pharyngitis, purulent skin lesions, and postinfectious sequelae. GAS strains display M protein on their surface and can be typed with antisera raised against the N-terminal portion of this α-helical coiled-coil molecule (467). More than 100 types are known, and it was recognized early that these type-specific antibodies conferred immunity to infection of strains carrying one specific M type by promoting the phagocytic killing of GAS (461, 467). Hence, much effort was directed at characterizing the M protein molecule as an antiphagocytic antigen (212).

In 1958, Jensen observed that several *S. aureus* strains could precipitate immunoglobulins (Ig) from both human and preimmune animal serum in gel precipitation assays (380). The nonimmune precipitation of antibodies is due to the binding of protein A on the staphylococcal surface to the Fc portion of Ig (222). However, antibodies directed specifically at protein A are not protective during animal infections, and strains deficient in protein A do not display a significant reduction in their pathogenic potential in an animal experimental system (403). Although we do not know the precise role of protein A during *S. aureus* infections, this molecule has served as a model system for immunological, structural, and microbiological studies (588).

It is widely assumed that surface proteins of Gram-positive bacteria might interact with eukaryotic proteins as a means of establishing residence at unique locations or evading the immune system. Although these assumptions have not always been rigorously tested, they have increased research interest in this field. The advent of molecular cloning techniques yielded a rapid accumulation of DNA sequence and allowed structural comparison of surface proteins. The current completion of several microbial genome sequences will soon provide us with a comprehensive view of all sequences. Therefore, we assume that future work will concentrate on the physiological and biochemical characterization of surface proteins in gram-positive bacteria.

Initial experiments to characterize surface proteins focused on solubilizing and purifying streptococcal M protein and staphylococcal protein A from the bacteria. Lancefield used acid extraction of streptococci to release M proteins from the cell surface (467). This treatment caused partial hydrolysis of polypeptides but not of the bacterial cell wall, and released peptide fragments were recovered in the supernatant after centrifugation of acid extracts. Treatment with detergents, bases, or proteases or by boiling also released some M protein; however, all of these preparations yielded either only peptide fragments or minute amounts of material, indicating that the solubilization technique was insufficient (212). This problem was overcome by the use of cell wall-hydrolytic enzymes, which enabled the efficient solubilization of full-length surface proteins (212, 340). The biochemical analysis of protein A was facilitated by its ability to be purified by affinity chromatography on matrices containing linked immunoglobulins after its enzymatic solubilization from the staphylococcal cell wall (340). When purified *S. aureus* cell walls were digested with lysostaphin, a bacteriolytic enzyme secreted by *Staphylococcus simulans* bv. staphylolyticus, protein A was released as a homogeneous population (737). Egg white lysozyme, an *N*-acetylmuramidase, does not effectively degrade the staphylococcal cell wall. Nevertheless, Sjöquist et al. were able to purify small amounts of lysozyme-released protein A and to show that it migrated more slowly on sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) than did the lysostaphin-released counterpart, suggesting an increase in molecular mass (738). Acid hydrolysis of the lysozyme-released species yielded the amino sugars GlcNAc and MurNAc, which were not observed for lysostaphin-released protein A (738). On the basis of these observations, Sjöquist et al. concluded that protein A must be linked to the staphylococcal cell wall (738).

Cloning and sequencing of the *emm* (streptococcal M protein) and *spa* (staphylococcal protein A) genes revealed open reading frames that specified polypeptides harboring an N-terminal signal peptide and a C-terminal hydrophobic domain (346, 803). Because hydrophobicity is a universal signal for membrane anchoring of polypeptides (138), it seemed plausible that surface proteins could be tethered to the cytoplasmic membrane of gram-positive bacteria rather than linked to the cell wall. Comparison of several additional surface protein gene sequences allowed a more detailed analysis of their primary structures and revealed a striking C-terminal homology, namely, the presence of an LPXTG sequence motif, where X is any amino acid, followed by a C-terminal hydrophobic domain and a tail of mostly positively charged residues (216). The remarkable conservation of the LPXTG motif in surface proteins of gram-positive bacteria suggested that this element must be involved in the anchor mechanism of surface proteins in gram-positive bacteria.

To characterize the C-terminal part of M proteins, Pancholi and Fischetti digested the surface exposed portion of this molecule with trypsin (613). Streptococci were washed to remove trypsin as well as cleaved peptide fragments and subsequently digested with phage lysin amidase, which resulted in a spectrum of released M-protein fragments with increasing mass when analyzed by SDS-PAGE (613). The fastest-migrating species was further purified and characterized by Edman degradation as well as amino acid composition. The data indicated that the C-terminal end of M protein, beginning at residue 302, is uniformly protease protected (613). Amino acid analysis revealed that the C-terminal peptide did not contain phenylalanine and suggested that the phenylalanine-containing hydrophobic domain might be absent from mature M protein (612). In a subsequent study of the possible membrane anchor cleavage activity, this data was interpreted as indicating a cleavage between the proline and the serine of the LPSTG motif (612), an estimate that was close to the later-identified cleavage site between the threonine and the glycine of the LPXTG motif within protein A (see below). Phage lysin released anchor fragments do not contain amino sugars (396). This can be explained by the amidase activity of this enzyme, which removes the glycan component of the cell wall from its crosslinked peptide backbone (613). Thus, although this has never been demonstrated, the data corroborate a model in which the C-terminal end of M proteins may also be covalently linked to the peptidoglycan.

Chemical analysis of the peptidoglycan of several other bacterial species including *S. pneumoniae* (476), *S. mutans*, *S. sanguis* (64, 680), *Lactobacillus fermenti* (822), and *Mycobacterium tuberculosis* (853) revealed the presence of significant amounts of nonpeptidoglycan amino acids. Analysis of the cell walls of *S. mutans* led to the conclusion that it contained covalently attached proteins, and it was suggested that this may be a feature common to the cell walls of all gram-positive species (577, 689).

SORTING: COVALENT LINKAGE OF SURFACE PROTEINS TO THE CELL WALL

Cell Wall Anchoring of Staphylococcal Protein A

Staphylococcal protein A has been used as a model system to study the anchoring of surface proteins in gram-positive bacteria (716). The cytoplasmic protein A precursor is exported and processed to generate the mature anchored species within 1 min of its synthesis. As described above, the anchored mature species is accessible to protease on the bacterial surface and requires enzymatic release from the staphylococcal cell wall for solubility. Lysostaphin cleaves at the pentaglycine crossbridge of the staphylococcal cell wall and solubilizes protein A as a uniform species on SDS-PAGE (716). In contrast, muramidase cleaves the glycan strands of the cell wall and muramidase-released protein A appears as a spectrum of bands on SDS-PAGE, all of which migrate more slowly than the lysostaphin-released counterpart (715). The notion that these mass differences must be the result of linked peptidoglycan was confirmed by an experiment in which muramidase-released protein A is digested with lysostaphin, which converts all material to the same uniform mobility as that observed for protein A directly solubilized by lysostaphin (715). Because of the uniform migration of lysostaphin-released fragments on SDS-PAGE, the anchoring point of surface proteins must be more proximal to the pentaglycine crossbridge, i.e., the cleavage site of lysostaphin, than to the glycan chains where muramidase cuts.

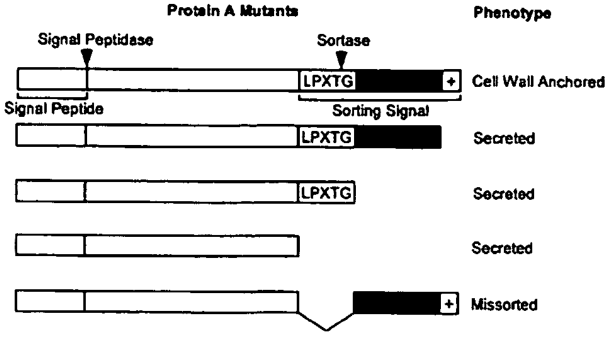


FIG. 8. Phenotypes of protein A cell wall sorting signal deletion mutants. Full-length protein A is found to be quantitatively anchored to the cell wall. Deletions of the charged tail, charged tail and hydrophobic domain, or the entire sorting signal result in the complete secretion of the polypeptide into the extracellular milieu. Deletion of the LPXTG motif of the C terminal abolishes the anchoring of the polypeptide to the cell wall. In the case of the protein A sorting signal, these mutant proteins are loosely associated with the cytoplasmic membrane.

Mutant Protein A Phenotypes

Protein A lacking a C-terminal sorting signal, i.e., the LPXTG motif, hydrophobic domain, and charged tail, is secreted into the medium and thus does not require the enzymatic digestion of the cell wall in order to be soluble when cells are boiled in SDS (716) (Fig. 8). Mutant protein A with the charged tail truncated behaves similarly. Lysostaphin-solubilized protein A migrates faster on SDS-PAGE than the secreted, unanchored species does, suggesting that the polypeptide chain is proteolytically cleaved during the anchoring process (716). To confirm that the secreted species comprised the complete (unprocessed) polypeptide chain, a protein A mutant that contains a single cysteine at the C terminus has been generated. Metabolic labeling of this cysteine revealed that the secreted protein A mutant is uncleaved and that some form of processing must be required for the anchoring of surface proteins (716).

Mutants with mutations within the LPXTG motif have a different phenotype. These polypeptides are not secreted into the medium but remain cell associated. Compared to the wild type, the LPXTG mutants do not reveal the characteristic size differences of lysostaphin- and muramidase-released species, indicating that these substances are not covalently linked to the cell wall (715). Furthermore, the mutant polypeptides also migrate more slowly on SDS-PAGE than their anchored counterparts do, suggesting that processing, i.e., proteolytic cleavage and cell wall linkage, is disrupted. Thus, the two different mutant phenotypes reveal that surface protein anchoring is arrested at distinct steps. The hydrophobic domain and charged tail cause protein A to be retained from the secretory pathway, whereas the LPXTG motif is needed for cleavage and linkage to the cell wall.

Cell Wall Sorting Signal

To determine the signal sufficient for cell wall anchoring of proteins normally secreted into the medium or lipid anchored to the cytoplasmic membrane, C-terminal protein A sequences have been fused to staphylococcal enterotoxin B (Seb), β-lactamase (BlaZ), or *E. coli* alkaline phosphatase (PhoA) exported by the protein A signal peptide (715, 716). The C-terminal 35 residues of protein A, comprising the LPXTG motif, hydrophobic domain, and charged tail, are sufficient for cell wall anchoring of each of these fusion proteins. Homologous sequence elements of other surface proteins also function to signal cell wall anchoring (715). When fused to the C terminus of secreted reporter proteins, some of these signals function in a manner indistinguishable from that of the protein A signal. Others fail to signal anchoring but can be mutated to gain this function. In all cases examined, these mutations alter the spacing between the LPXTG motif and the charged tail. The absolute number of residues between the LPXTG motif and the charged tail does not appear to be critical. It has been proposed that the folding of the hydrophobic domain determines the correct spacing between the flanking elements required for proper recognition of the sorting signal (715).

Retention is signaled by the positively charged residues within the charged tail of the sorting signal. Although a single arginine is most effective in signaling retention, this residue can be replaced by lysine when other positively charged residues are present. Increasing the spacing between the LPXTG motif and the charged tail further does not interfere with cell wall sorting but proves to be toxic for staphylococci. The reason for this phenomenon is not known (715).

The LPXTG motif is conserved within the sorting signals of all known wall-anchored surface proteins of gram-positive bacteria (Table 1). The threonine (T) displays some variation in that either alanine or serine can be found at this position. A threonine-to-alanine substitution was tested in the sorting signal of staphylococcal protein A, and this mutation does not affect anchoring, which suggests that the identified sequences are indeed functional sorting signals. Other mutations in the LPXTG motif, for example a proline (P)-to-asparagine (N) mutation, do abolish sorting. Nevertheless, a rigorous mutagenesis of this sequence element has never been performed, and the basis for the sequence conservation is thus unknown. The simplest explanation for the conservation of the LPXTG motif may be the preservation of a proteolytic cleavage site. Sortase, the presumed enzymatic activity that cleaves at this point and catalyzes the transpeptidation reaction, may require a specific sequence or fold for substrate recognition. It is surprising that other transpeptidation mechanisms, for example that of glycosylphosphatidylinositol (GPI) anchoring, seemingly have much less stringent substrate requirements (see below).

The hydrophobic domain and charged tail together are thought to retain the polypeptide from the secretory pathway and provide an opportunity for the proteolytic cleavage of surface proteins. One way in which this might be achieved is to anchor the polypeptide in the cytoplasmic membrane. If so, fusion of the C-terminal hydrophobic domain to other secreted proteins such as PhoA or Seb should insert the hybrids in the membrane by acting as a stable transmembrane domain. This, however, is not the case, and the mutant proteins are instead found to be peripherally associated with the membrane (715). Furthermore, mutation or truncations of the charged tail, even at positions that cannot play a role in membrane insertion, lead to the secretion of the polypeptide into the surrounding medium. Perhaps the C-terminal hydrophobic domain and charged tail function to arrest translocation of the polypeptide within the secretion channel but without permitting the actual insertion into the membrane. This arrest in translocation could suffice to allow cleavage of the polypeptide and concomitant cell wall anchoring. A simple test of this hypothesis might be to engineer sorting signals with increased hydrophobicity to allow membrane anchoring. Once inserted into the membrane, the surface protein could diffuse away from the site of cell wall sorting without permitting a transpeptidation reaction at the LPXTG motif.

Proteolytic Cleavage at the LPXTG Motif

The proteolytic cleavage site during the anchoring of surface proteins has been determined by purifying and sequencing the C-terminal cleavage fragment (574). This has been done by using a hybrid protein in which the mature part of maltose binding protein is fused to the C-terminal end of the sorting signal. The hybrid protein is exported by its N-terminal signal peptide and cleaved, and the N-terminal portion is linked to the bacterial cell wall. In contrast, the C-terminal cleavage fragment with the fused maltose binding protein domain remains within the cytoplasm and can be purified by affinity chromatography (574). Edman degradation reveals that the cleavage site is located between the threonine and the glycine of the LPXTG motif (Fig. 9). Deletion of the LPXTG motif abrogates cleavage as well as anchoring, indicating that the two events are linked. Proteolytic cleavage at the LPXTG motif absolutely requires protein export by the Sec machinery. When cells are poisoned with sodium azide, which at low concentrations is an inhibitor of the SecA motor of preprotein translocase (223, 601), no secretion or cleavage of the fusion protein occurs. Similarly, protein A mutants harboring a defective signal peptide are not cleaved at the LPXTG motif. This result suggests that the site of proteolytic cleavage may be either in the membrane, i.e., during secretion of protein A, or on its extracytoplasmic side, which coincides with the locus for cell wall assembly.

Cell Wall Anchor Structure of Protein A

To date, the only cell wall anchor structure to be solved in molecular detail is that of staphylococcal protein A (575, 713, 796, 797). The structure was determined through the use of specifically designed hybrid proteins that enabled the purification of large amounts of surface protein after solubilization from the cell wall with muralytic enzymes. The C-terminal anchor structure was removed and isolated from the full-length hybrid surface protein by proteolysis or cyanogen bromide cleavage at sites specifically engineered into the hybrid protein. The resulting C-terminal anchor peptides allowed detailed studies of associated cell wall structures by chemical analysis, Edman degradation, and mass spectrometry.

In an early study, a maltose binding protein harboring the C-terminal sorting signal of protein A was solubilized with lysostaphin, purified, and subjected to limited proteolysis to identify C-terminal anchor peptides (713). The C-terminal threonine of the LPXTG motif was found to be amide linked to a triglycine peptide. Because the pentaglycine crossbridge of the staphylococcal cell wall is also the site of lysostaphin cleavage, it was proposed that surface proteins are amide linked to the free amino group of the wall crossbridges. Because both the LPXTG motif and the free amino group of wall crossbridges are conserved features in gram-positive bacteria, this mechanism might be universal for all organisms expressing surface proteins via a C-terminal sorting signal (574, 713).

In later studies, a hybrid protein containing the C-terminal sorting signal of protein A fused to Seb was used in similar experiments to analyze the C-terminal anchor structure of surface protein solubilized with several other muralytic enzymes (575, 796, 797). In these studies, six histidyl residues placed at the fusion junction between Seb and the sorting signal facilitated the purification of the full-length protein by chromatography on nickel resin. C-terminal anchor peptides were ob-

TABLE 1. Proteins containing cell wall sorting signals

Source	Sequence of C-terminal sorting signal[a]	Reference(s)
Actinomyces naeslundii		
Fim P: type 1 fimbrial subunit (T14V)	LPLTGANGVIFLTIAGALLVAGGAVVAYANKRRHVAKH	868
Fim A: type 1 fimbrial subunit (T14V)	LPLTGANGMLILTASGASLLMIAVGSVLVARYRERKQNANLAL	869
Type 2 fimbrial subunit (WVU45)	LPLTGANGMLILTASGAALLMIAVGSVLVARYRERKRNRDLAA	867
Enterococcus faecalis		
Asa1: aggregation substance	LPQTGEKQNVLLTVAGSLAAMLGLAGLGFKRRKETK	237
Asc10: aggregation substance	LPKTGEKQNVLLTVVGSLAAMLGLAGLGFKRRKETK	411
Asp1: aggregation substance	LPHTGEKENTLLSVLGAGMLAGLAWFGLKRKETEK	236
Sea1: surface exclusion protein	LPHTGEQKSIWLTIFGLFMAVGAISFKNKRRKNS	840
Sec10: surface exclusion protein	LPQTGEQQSIWLTIIGLLMAAGTINFKNKKRKKNS	411
PrgC: phermone-responsive surface protein	LPHTGEKFTLLFSVLGSFFVLISGFFFFKKNKKKA	411, 571
Unknown ORF[b] (hypothetical 30.5 kDa)	LPKTGETENIALSVLGSLMVLGSAFIFKKRI	771
Enterococcus faecium		
Ash701: aggregation substance	LPQTGEKQNILLTVAGSLVTMLGLAGVGLKRRKETK	315
Lactococcus lactis		
CluA: sex factor aggregation protein	LPKTGEGKAALAISIFGAALLGLAAYLKRNWIVSTYRKTVRKIRK	271
NisP: nisin serine protease	LPVTGDGEDFLPALGIVCISILGILKRKTKN	810
PrtP: casein serine protease	LPKTGETTERPAFGFLGVIVVSLMGVLGLKRKQREE	820
Lactobacillus paracasei		
PrtP: casein serine protease	LPKTAETTERPAFGFLGVIVSLMGVLGLKRKQREE	343
Listeria monocytogenes		
Internalin A	LPTTGDSDNALYLLLGLLAVGTAMALTKKARASK	235
Internalin C2	LPTAGDENTMLPIFIGVFLLGTATLILRKTIKVK	162
Internalin D	LPTAGDENTMLPIFIGVFLLGTATLILRKTIKVK	162
Internalin E	LPITGDELNVLPIFVGAVLIGIGLVLFRKKRQTK	162
Internalin F	LPKTGDNAPWKTLFAGILLSSSAFYIWRKKA	162
Peptostreptococcus magnus		
Protein L	LPKAGSEAEILTLAAAALSTAAGAYVSLKKRK	412, 567
PAB	LPEAGRRKAEILTLAAASLSSVAGAFISLKKRK	141
Streptococcus pyogenes (GAS)		
C5A peptidase	LPTTNDKDTNRLHLLKLVMTTFFFGLVAHIFKTKRQKETKK	118
Trypsin-resistant surface protein (T6)	LPSTGSIGTYLFKAIGSAAMIGAIGIYIVKRRKA	714
SfbI/protein F	LPATGDIENVLAFLGILILSVLSIFSLLKNKQSNKKV	720, 778
SfbII/OF	LPASGDKREASFTIVALTIIGAAGLLSKKRRDTEEN	448, 658
M protein		
Class I Emm (M6)	LPSTGETANPFFTAAALTVMATAGVAAVVKRKEEN	Table 2
Class II Emm (Emm22)	LPSTGEAANPFFTAAAATVMVSAGMLALKRKEEN	Table 2
Mrp/FcrA (Mrp8)	LPSTGEETTNPFFTAAALTVIASAGVLALKRKEEN	Table 2
Enn (Enn49)	LPSTGEAANPFFTAAAATVMVSAGMLALKRKEEN	Table 2
Unknown ORF	LPSTGEKAQPLFLATMTLMSLLGSLLVTKRQKETKK	639
Streptococcus agalactiae (GBS)		
Alpha antigen	LPATGENATPFFNVAALTIISSVGLLSVSKKKED	550
Rib protein	LPATGENATPFFNVVALTIMSSVGLLSVSKKKED	837
Beta antigen (IgA Fc receptor)	LPYTGVASNLVLEIMGLLGLIGTSFIAMKRRKS	319, 382
C5a peptidase	LPTTNDKDTNRLHLLKLVMTTFFLGLVAHIFKTKRQKETKK	119
Streptococcus sp. (GCS and GGS)		
Protein G (IgG binding)	LPTTGEGSNPFFTAAALAVMAGAGALAVASKRKED	194, 290
M protein (Group C)	LPSTGEATNPFFTAAALAVMATAGVAAVVKRKEEN	776
MIG (strain SC1)	LPTTGEGSNPFFTAAALAVMAGAGALAVASKRKED	401
MAG (strain 8215)	LPTTGEGSNPFFTAAALAVMAGAGALAVASKRKED	399
FnBA: fibronectin binding protein	LPQTGDTNKLETFFTITALTVIGAAGLLGKKRRNNQTD	490
FnBB: fibronectin binding protein	LPAAGEAEHVLSTIVGAMILFLVSLWGLLKRKASKA	490
GfbA: fibronectin binding protein	LPATGDIENVLAFLGILILSVLPIFSLLKNKQNNKV	430
Streptococcus gordonii		
SspA/SSP-5 (antigen I/II)	LPKTGTNDSSYMPYLGLAALVGVLGLGQLKRKEDESK	149
SspB (antigen I/II)	LPKTGTNDATYMPYLGLAALVGFLGLGLAKRKED	148, 149
CshA	LPRTGSQTSDQTASGLLAAIASLTFFGLANRKKKSKED	538, 539

Continued on following page

TABLE 1—*Continued*

Source	Sequence of C-terminal sorting signal[a]	Reference(s)
Streptococcus mutans		
Antigen I/II (P1, B, PAc)	**LPNTG**VTNNAYMPLLGIIGLVTSFSLLGLKAKKD	422, 423, 598
β-D-Fructosidase	**LPDTG**DHKTDLSQLGVLAMIGSFLVEIASYFKKSKD	103
Dextranase	**LPQTG**DNNETRSNLLKVIGAGALLIGAAGLLSLIKGRKND	363
Glucan binding protein C	**LPHTG**AAKQNGLATLGAISTAFAAATLIAARKKEN	704
WapA	**LPSTG**EQAGLLLTTVGLVIVAVAGVYFYRTRR	201
Streptococcus pneumoniae		
β-*N*-Acetylhexoseaminidase	**LPETG**THDSAGLVVAGLMSTLAAYGLTKRKED	122
Neuraminidase	**LPETG**NKESDLLASLGLTAFFLGLFTLGKKREQ	107
Streptococcus sobrinus/downeii		
SPAA (antigen I/II; PAg)	**LPATG**DSSNAYLPLLGLVSLTAGFSLLGLRRKQD	794
Dextranase	**LPKTG**DHKTVVLIIVLGLVFVGMTGLLARHEKK	827
Streptococcus equi		
ZAG	**LPTTG**EKANPFFTAAALAIMAGAGALAVTSKRQQD	400
Fibronectin binding protein	**LPQTS**DMKQLTLSIIGAMSMLLVLCLSLFKRPSKKD	491, 492
SeM	**LPSTG**ESANPFFTIAALTVIAGAGMAVVSPKRKEN	784
SzPW60	**LPSTG**EATNPFFTAAALAVMAGAGVAAVSTRRKEN	785
SzPSe	**LPSTG**EATNPFFTAAALAVMAGAGVAAVSTRRKEN	784
Streptococcus suis		
136-kDa muramidase released surface protein	**LPNTG**EASSVAGALGTAMLVATLAFARKRRRNED	747
Staphylococcus aureus		
Collagen adhesin	**LPKTG**MKIITSWITWVFIGILGLYLILRKRFNS	626
Fibrinogen binding protein (clumping factor)	**LPDTG**SEDEANTSLIWGLLASIGSLLLFRRKKENKDKK	531
FNBP: fibronectin binding protein	**LPETG**GEESTNKGMLFGGLFSILGLALLRRNKKNHKA	727
Protein A: IgG binding protein	**LPETG**EENPFIGTTVFGGLSLALGAALLAGRRREL	726, 803
Staphylococcus epidermidis		
Fibrinogen binding protein	**LPDTG**ANEDYGSKGTLLGTLFAGLGALLLGKRRKNRKNKN	590

[a] The LPXTG motif is shown in boldface type.

[b] ORF, open reading frame.

tained as follows. The purified surface protein was first cleaved with cyanogen bromide at a methionyl immediately adjacent to the six histidyl. The anchor peptides were then purified by another round of affinity chromatography on nickel resin. As mentioned above, surface protein solubilized from staphylococcal peptidoglycan with muramidases migrate as a ladder of bands on SDS-PAGE (Fig. 10). Muralytic amidases, i.e., enzymes that cut at the amide linkage between the D-lactyl of MurNAc and the L-Ala of the wall peptide, also solubilize surface protein as a ladder of bands (575). Mass-spectrometric analysis of C-terminal anchor peptides from the hybrid surface protein solubilized with these enzymes confirmed that the distinct pattern observed on SDS-PAGE is due to the attachment of a variable number of linked peptidoglycan subunits (575). Amidase-solubilized surface proteins were found to be linked to one or more peptidoglycan subunits of the structure [NH_2–L-Ala–D-iGlu–L-Lys(Gly_5)–D-Ala–D-Ala], whereas the muramidase-solubilized surface proteins were linked to subunits of the structure MurNAc[-L-Ala–D-iGlu–L-Lys(Gly_5)–D-Ala–D-Ala]-GlcNAc. The attached peptidoglycan subunits were found to be linked to one another through their pentaglycine crossbridges. As would be expected, redigestion of amidase- and muramidase-solubilized surface protein with lysostaphin removed the linked peptidoglycan subunits (575). The structure of a surface protein linked to a peptidoglycan dimer is shown in Fig. 11.

On the basis of sequence homology and limited biochemical analysis, the murein hydrolase of staphylococcal bacteriophage φ11 was predicted to be an amidase (835). Hybrid surface proteins solubilized from the staphylococcal cell wall with the φ11 hydrolase migrate as a doublet of bands on SDS-PAGE, in marked contrast to the ladder pattern observed when the protein is solubilized with other well-characterized amidases (796) (Fig. 10). Analysis of φ11 hydrolase-solubilized C-terminal anchor peptides revealed that surface proteins were linked to a single peptidoglycan subunit, of which approximately 50% lacked the GlcNAc-MurNAc disaccharide, indicating that cross-linked peptidoglycan subunits had been removed (796). Recent results reveal that the φ11 hydrolase displays two activities, the expected amidase as well as a D-Ala-Gly peptidase activity (575, 576).

Peptidoglycan Substrate of the Sorting Reaction

Genetic analysis of staphylococcal methicillin resistance has provided new insights into the synthesis of the peptidoglycan crossbridge (43). Staphylococcal strains expressing PBP2a (PBP2′) are resistant to most β-lactam antibiotics including methicillin (310, 520). Genetic screens designed to identify other elements necessary for methicillin resistance yielded mutations in at least 10 different genes (44, 45, 146). Some of these genes are involved in the synthesis of the pentaglycine crossbridge (145, 331, 513, 756) or the amidation of D-isoglutamyl within the wall peptide (292, 394). Staphylococcal strains harboring mutations in the *femA*, *femB*, or *femX* gene synthesize altered cell wall crossbridges with either three glycyl

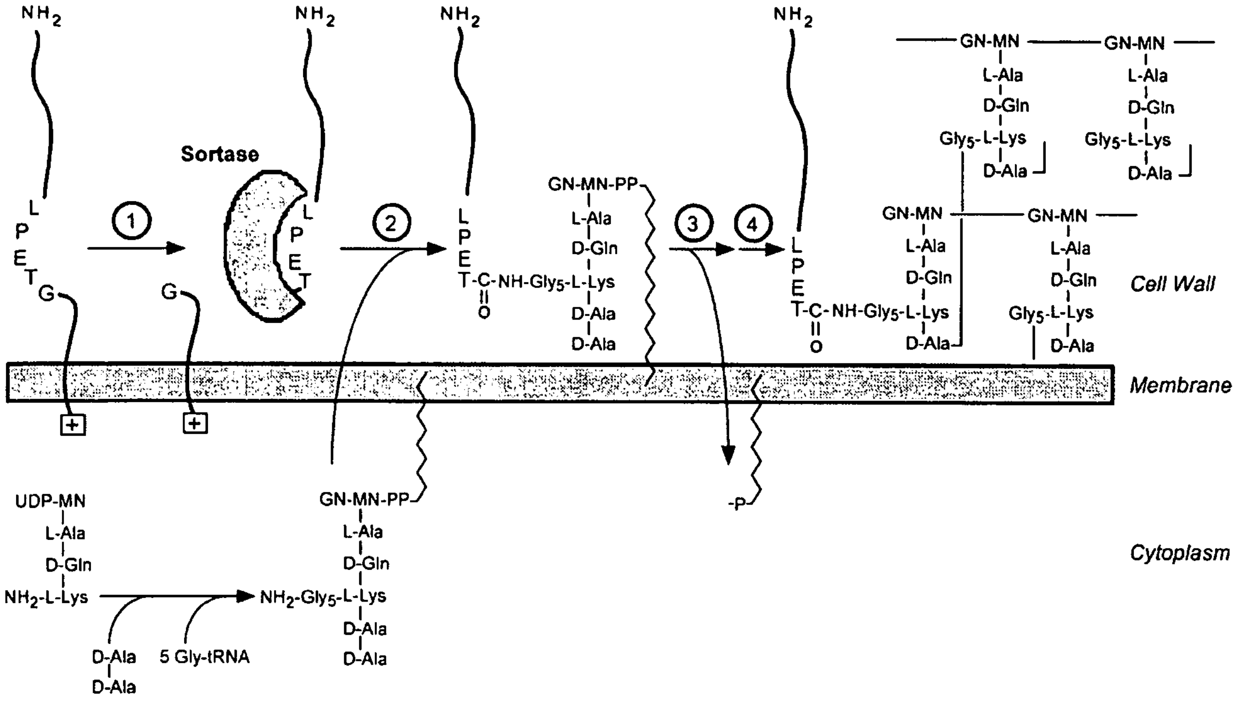


FIG. 9. Proposed model for the cell wall sorting reaction. The model can be divided into four distinct steps. 1, the full-length precursor is exported from the cytoplasm via an N-terminal leader peptide. 2, the protein is prevented from release into the extracellular milieu by the charged tail and hydrophobic domain. 3, the protein is cleaved between the threonyl and glycyl residues of the LPXTG motif. 4, the newly liberated carboxy terminus of threonine is transferred via an amide bond exchange to an amino group found at the end of the wall crossbridge. In this proposed model, steps 3 and 4 are coupled in a two-step reaction. Overall, the reaction bears similarity to the amide bond exchange mechanisms by which proteins are attached to GPI moieties and by which transpeptidation occurs during cell wall synthesis.

(*femB*), one glycyl (*femA*), or a combination of no or one glycyl. The last phenotype has been reported for a mutant with a combination of a *femA* mutation and a second one leading to a partially nonfunctional FemX protein (442). Although this has not yet been demonstrated directly, it seems likely that FemA, FemB, and presumably also FemX catalyze the addition of glycyl to the ε-amino group of L-lysyl in the wall peptide portion of lipid II. Ton-That et al. tested whether the sorting reaction could proceed in staphylococcal strains carrying mutations in any of the three genes *femA*, *femB*, and *femX* (797). Although sorting was slowed in all *fem* mutant strains, surface protein was found anchored to peptidoglycan-bearing crossbridges with only three or one glycyl residues as well as crossbridges containing seryl moieties. However, species anchored directly to the ε-amino of L-lysyl in the wall peptide were not observed, suggesting that the sorting reaction has restricted substrate specificity for the amino group acceptor of this amide bond exchange mechanism (797).

Although it is now clear that protein A and other staphylococcal surface proteins are linked to the pentaglycine cell wall crossbridge, the specific peptidoglycan substrate of the sorting reaction has thus far not been identified. If the sorting reaction requires mature, assembled peptidoglycan as a substrate, staphylococcal protoplasts in which the peptidoglycan had been removed enzymatically should be unable to catalyze the cleavage of sorting precursors. Movitz studied the synthesis of protein A in staphylococcal protoplasts and reported that most of this polypeptide was released into the extracellular medium (561). A similar result was observed for the secretion of M protein by streptococcal protoplasts (809). Staphylococcal protoplasts generated by lysostaphin digestion and osmotically stabilized with sucrose cleaved sorting precursors similarly to

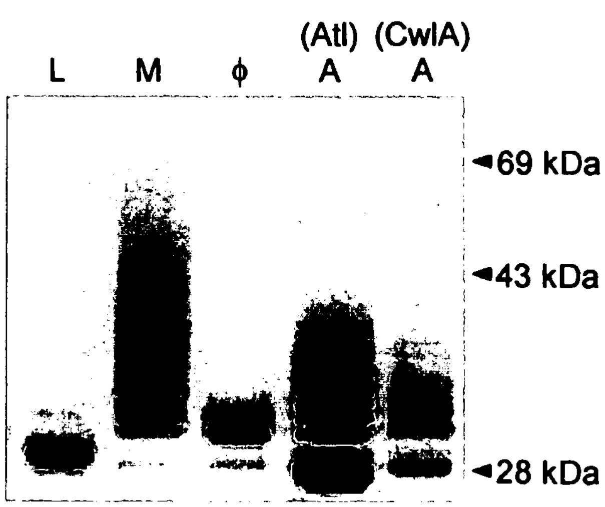


FIG. 10. SDS-PAGE of surface protein solubilized with five different muralytic enzymes. Shown is a Coomassie blue-stained SDS-PAGE gel of a hybrid surface protein containing the cell wall sorting signal of protein A after solubilization from the staphylococcal peptidoglycan with lysostaphin (L), mutanolysin (M), the staphylococcal phage φ11 murein hydrolase (φ), the amidase portion of the major staphylococcal autolysin (A, Atl), or the lytic amidase from *B. subtilis* (A, CwlA). Proteins solubilized with these enzymes display distinct mobilities on SDS-PAGE due to the presence of covalently linked cell wall subunits (see the text). See Fig. 17 for a diagram of the enzymatic activities of these enzymes. A species of 28 kDa is apparent in the amidase-, phage hydrolase-, and mutanolysin-digested samples, which migrates faster than the lysostaphin-solubilized protein. The generation of this species is apparently due to degradation during the prolonged incubation necessary for complete solubilization of the cell wall with these enzymes. Reprinted from reference 575 with permission of the publisher.

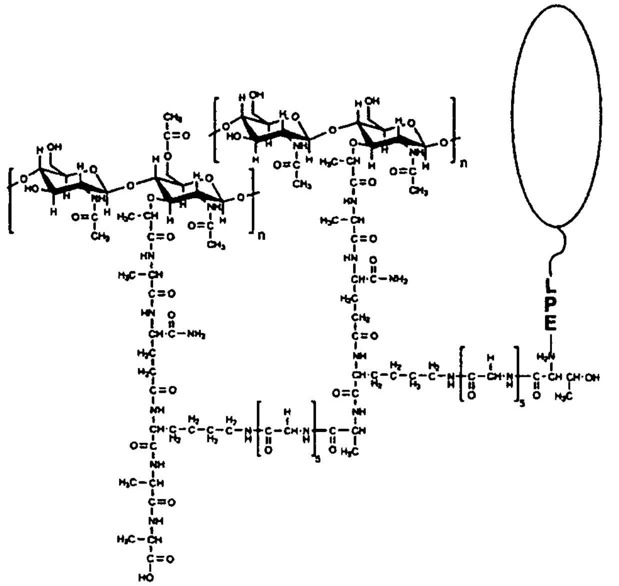


FIG. 11. Structure of a surface protein linked to the peptidoglycan of *S. aureus*. As shown here, the protein is linked to a wall subunit that has been incorporated into the peptidoglycan network by both transpeptidation and transglycosylation. The neighboring wall subunit exists as a pentapeptide and is therefore not substituted. Adapted from reference 575 with permission of the publisher.

intact cells, suggesting that the mature, assembled cell wall is not required for the sorting reaction (797a). Protein A molecules that were cleaved at the LPXTG motif were released into the extracellular medium. Nevertheless, it is not clear whether these released protein A molecules contain linked peptidoglycan fragments.

On the other hand, if the sorting reaction uses the peptidoglycan synthesis precursor, lipid II, as a substrate, inhibition of peptidoglycan synthesis with known antibiotics might also interfere with the sorting reaction. This question has also been addressed by Movitz (562). Although the anchoring mechanism was unknown at that time, the author suspected that protein A was linked to peptidoglycan and measured the amount of pulse-labeled protein A in the cell walls of staphylococci that had been treated with vancomycin. The results showed that vancomycin inhibited cell wall synthesis but did not interfere with the anchoring of protein A. Movitz concluded that protein A may be linked to mature, assembled cell wall. Vancomycin binds to D-alanyl–D-alanine within the wall peptide, and treatment of staphylococci with this antibiotic leads to the accumulation of lipid II molecules (789). If lipid II served as substrate for the sorting reaction, Movitz's experiments could not have distinguished between protein A molecules that were linked to lipid II and others that were tethered to the assembled cell wall. We think that the identification of the substrate for the sorting reaction will require biochemical studies with purified sortase enzyme as well as the rigorous characterization of presumed sorting intermediates.

Cell Wall Sorting Mechanism: a Model

The sorting of surface proteins to the cell wall of gram-positive bacteria begins in the cytoplasm with the initiation of precursor molecules into the protein export (Sec) pathway via their N-terminal leader peptides (Fig. 9). By default, this pathway leads to the secretion of polypeptides into the surrounding medium of staphylococci; however, the C-terminal hydrophobic domain and charged tail function to retain protein A along the pathway, perhaps within the preprotein translocase. This retention allows recognition of the LPXTG motif and a proteolytic cleavage between the threonine and the glycine of the LPXTG motif. The carbonyl of threonine may be acylated to an active-site thiol or hydroxyl of sortase, and a subsequent nucleophilic attack of the free amino at the end of the pentaglycine cell wall crossbridge results in the transpeptidation of surface protein to peptidoglycan precursor. The sorting intermediate may subsequently be incorporated into the cell wall. Proteolytic degradation of anchored proteins as well as the physiological turnover of peptidoglycan will finally remove the anchored polypeptides.

Murein Lipoprotein of Gram-Negative Bacteria: a Comparison

Braun's murein lipoprotein (LPP) serves as a paradigm for the synthesis and structure of cell wall-linked proteins in gram-negative bacteria (85) (Fig. 12). The N-terminal signal peptide of LPP directs the polypeptide into the secretory pathway (367). A cysteine residue within the signal peptide is glyceride modified via a thioester linkage prior to proteolytic cleavage at its amino group (305). The amino group of the N-terminal cysteine is acylated prior to the insertion of LPP into the outer membrane (485). The 70-residue polypeptide of mature LPP trimerizes in the periplasmic space (84, 87). About one-third of all lipoprotein is covalently linked to the peptidoglycan layer, thereby tethering the outer membrane to the peptidoglycan skeleton of *E. coli* (86). Lipid modification of murein lipoprotein first requires membrane-embedded glyceryl transferase and *O*-acyl transferase activities (240, 289, 783). The glycerol-modified LPP precursor is then cleaved by signal peptidase (type II), and the liberated amino group of cysteine is modified by an *N*-acyl transferase (365). The ε-amino of the C-terminal lysine is amide linked to the carboxyl group at the D center of *meso*-diaminopimelic acid within the *E. coli* cell wall peptides (88, 89). Neither the chemical reaction nor the enzymes responsible for this linkage have been characterized. It is conceivable that each trimeric murein lipoprotein unit is covalently linked to the cell wall. It also seems plausible that the amide bond between LPP and cell wall is generated at the expense of another amide bond, namely, that between *meso*-diaminopimelic acid and D-Ala in cell wall tetrapeptides, similar to penicillin-sensitive transpeptidation and the sorting mechanism of surface proteins anchored to the cell wall of gram-positive bacteria.

Amide Linkage of Eukaryotic Surface Proteins to GPI Anchors: a Comparison

Some proteins displayed on the apical surface of polarized eukaryotic cells are modified by the GPI anchor (187, 198, 527) (Fig. 13). These polypeptides are synthesized with an N-terminal signal peptide and a C-terminal hydrophobic domain that functions to transiently retain these polypeptides in the endoplasmic reticulum (ER) membrane. Precursors are recognized in the ER as substrates by a GPI-modifying enzyme which proteolytically cleaves the peptide backbone upstream of the hydrophobic domain and amide links the N-terminal cleavage fragment to the free amino group of ethanolamine within the GPI moiety (69). GPI-decorated surface protein species are thought to be sorted at the Golgi and *trans*-Golgi network to lipid vesicles containing ceramide rafts that are marked for fusion with the apical portion of the cytoplasmic membrane

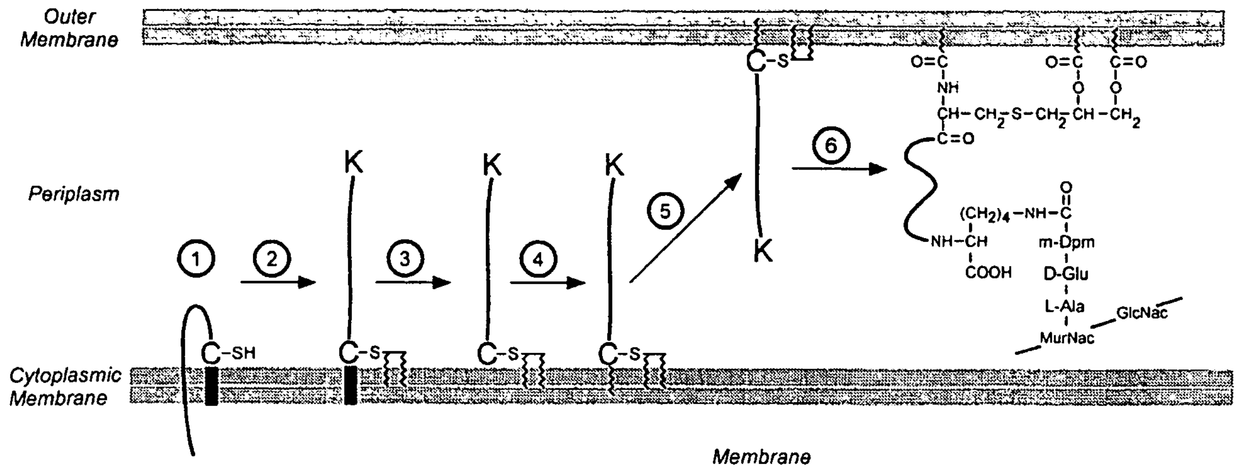


FIG. 12. Lipid and peptidoglycan attachment of the murein (Braun's) lipoprotein of *E. coli*. The complete processing of the lipoprotein occurs in several discrete steps. 1, The protein is exported from the cytosol by virtue of a type II N-terminal leader peptide. 2, The cysteine residue of the leader peptide is modified by the addition of diacylglycerol. 3, The leader peptide is removed by the type II leader peptidase. 4, The newly liberated amino terminus is acylated. 5, The protein is translocated to the periplasmic side of the outer membrane. 6, The C-terminal lysine residue of the lipoprotein is amide linked to a carboxyl group of the diaminopimelic acid at position 3 of a wall peptide. Like the surface proteins of gram-positive bacteria, the lipoprotein is amide linked to the peptidoglycan. However, in this case the cell wall donates the carboxyl group rather than the amino group to this bond.

(96, 495, 674). The exact mechanism of vesicle targeting is not known; however, the apical membrane differs in lipid composition from the basolateral membrane, which does not contain sphingomyelin and cerebrosides (203, 874). GPI-anchored surface proteins can be internalized by the budding of surface lipids into a structure referred to as caveoli (421). These vesicles are destined for fusion with lysosomes, which results in the degradation of both the GPI-anchored proteins and their bound ligands (522).

Synthesis of the GPI moiety occurs in the ER and begins on the cytoplasmic leaflet of the membrane (523, 524, 526, 813). After linkage of GlcNAc to phosphatidylinositol, a three-mannose core glycan is added and capped with phosphoethanolamine. The GPI moiety can translocate to the luminal side of the ER membrane and then serve as a substrate for transamidation (187, 775) (Fig. 14). The requirements for substrate recognition of precursor proteins have been thoroughly investigated (248, 434, 435, 548, 549, 556, 557). No conserved amino acids are present at or near the cleavage site, a situation more reminiscent of N-terminal leader peptides than of C-terminal cell wall sorting signals. Nevertheless, extensive mutagenesis of this region revealed that the residue at the cleavage site (ω) could be glycine, alanine, cysteine, serine, or asparagine but was none of the other 15 amino acids found in proteins. The ω+1 position, i.e., the residue at the N terminus of the C-terminal cleavage fragment, could tolerate almost all substitutions except proline. However, ω+2 appears to require the presence of either glycine, serine, threonine, or alanine, i.e., small-chain and uncharged amino acids (435). Furthermore, although the hydrophobic domains of GPI-modified proteins suffice to transiently insert precursors into the membrane, these sequences are also not characteristic membrane anchors and often lack the terminal charged residues as well as the degree of hydrophobicity found in peptide membrane anchors. Their function may therefore be similar to that of the C-terminal hydrophobic domain and charged tails of gram-positive sorting signals, i.e., to temporally retain the polypeptide chains from the secretory pathway and to permit a C-terminal transpeptidation reaction. Single point mutations within the hydrophobic domain suffice to stabilize the peptide membrane anchor and allow insertion into the basolateral portion of the cytoplasmic membrane (828).

Genes encoding the enzymes involved in synthesis of the GPI anchor were identified by screening for yeast mutants defective in the incorporation of [^{3}H]inositol into the GPI-anchored protein GAS1 (604). Mutants with mutations in three genes, *gpi-1*, *gpi-2*, and *gpi-3*, are defective in GPI anchoring and temperature sensitive for growth, suggesting that these genes may be essential. Strains carrying mutations in any of three genes were found to be defective in the first step of

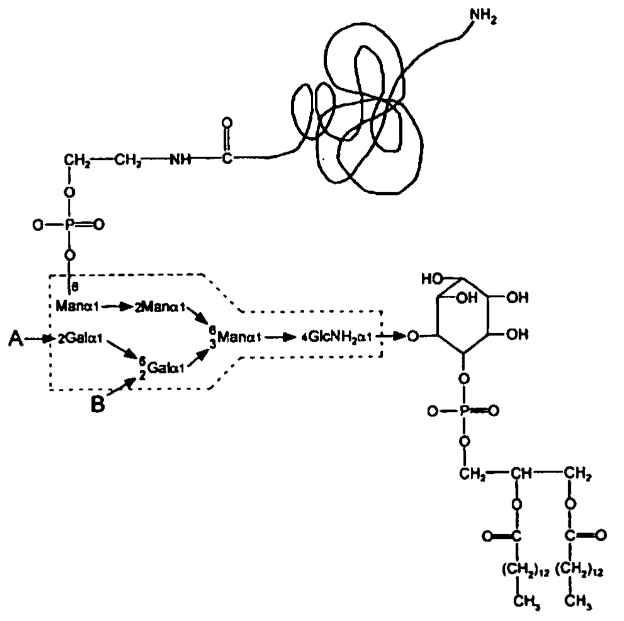


FIG. 13. Core structure of a GPI anchor. This diagram shows the core GPI anchor structure found to be common among almost all known GPI anchors from several species. Species-specific variation of the anchor structure can occur through the addition of glycan moieties that can be attached at the points labeled A and B.

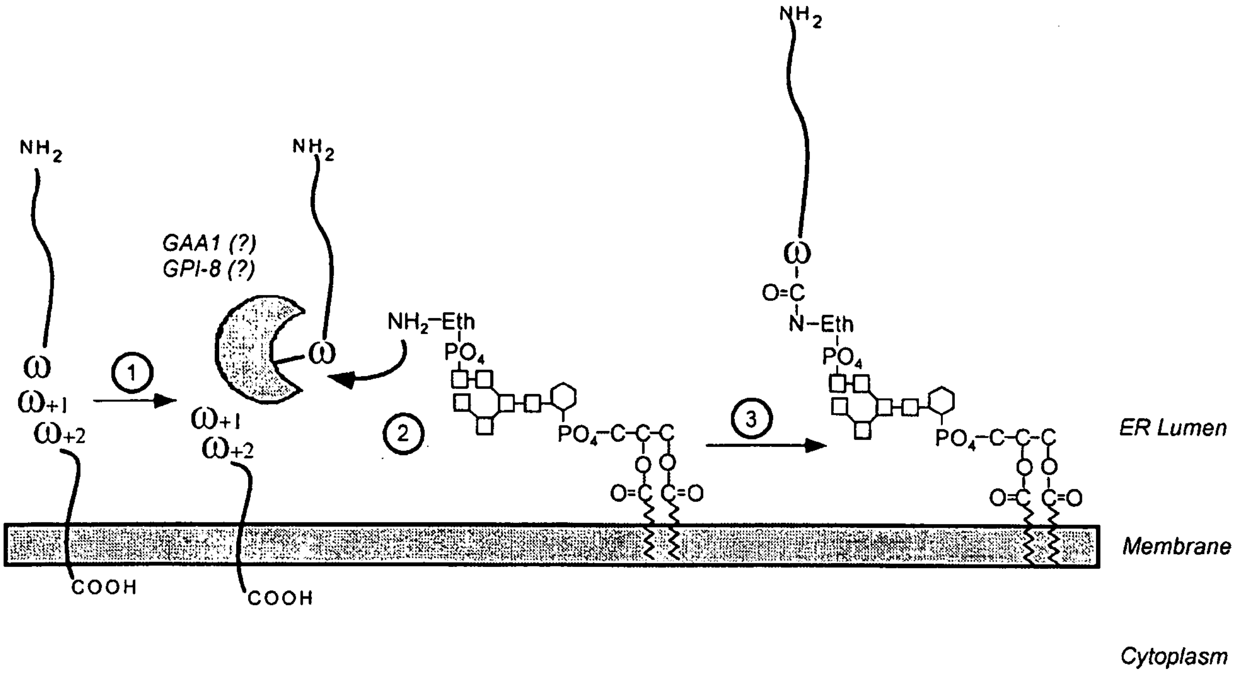


FIG. 14. Attachment of a GPI anchor to the C-terminal end of a polypeptide chain. 1, polypeptides entering the secretory pathway are transiently anchored to the membrane of the ER via the hydrophobic portion of the C-terminal GPI-anchoring signal. 2, cleavage occurs between the ω and ω+1 residues. 3, the newly liberated carboxy-terminus of the ω residue is amide linked to the amino group of the ethanolamine of the GPI anchor. This mechanism is similar to the mechanism by which cell wall sorting of surface proteins is proposed to occur in gram-positive bacteria.

GPI anchoring, the transfer of UDP-GlcNAc to phosphatidylinositol (478–480, 838). A second approach screened for yeast mutants defective in the surface presentation of GPI-anchored agglutinin, and the identified mutants could be grouped into six complementation classes, *gpi-4* to *gpi-9* (33). One of those strains, carrying a mutant *gpi-8* allele, synthesizes a complete GPI moiety but does not transfer it onto precursor proteins, whereas all other mutants are defective in GPI assembly (32). GPI8 is a type I transmembrane ER protein with homology to cysteine proteases (32). Another transmembrane ER protein, GAA1, is also required for the attachment of GPI anchors to polypeptides (300, 338). Furthermore, it has been reported that soluble ER components are also necessary for the anchoring process (814). The role of these factors in catalyzing the transpeptidation reaction of GPI anchoring will have to be determined in a biochemical assay.

In vitro GPI anchoring of precursor protein has been demonstrated independently by several different laboratories (196, 525, 802). Mayor et al. established an assay that allows measurements of GPI anchoring without protein translocation into microsomal membranes (525). This is by far the simplest system and should allow characterization of the required enzymatic activities. The in vitro reaction requires precursor protein and GPI but no ATP or GTP, consistent with a transpeptidation (transamidation) mechanism. It is conceivable that the GPI8 enzyme catalyzes this reaction via an active-site sulfhydryl; a definitive result should be forthcoming.

Sorting at a Distinct Site?

Cole and Hahn examined the sites of cell wall and surface protein synthesis in *Streptococcus pyogenes* (127). This gram-positive organism forms chains of coccal cells due to the incomplete separation of its peptidoglycan. Thus, although the individual cells are round, the chain appearance permits an easy orientation of cell division sites at the contact points between cells. New cell wall appeared to be synthesized only at the contact points between cells. This experiment was extended to the synthesis of surface proteins by first proteolytically removing all surface (M) proteins and detecting the newly synthesized polypeptide by immunofluorescent staining of type-specific antiserum. All newly synthesized M protein first appeared at the contact sites between streptococcal cells and then extended slowly over the entire surface of the gram-positive cell. This result suggests that streptococci incorporate their surface proteins together with peptidoglycan at a defined site. Such localization may be required to coordinate protein sorting and cell wall synthesis, but these sites could also represent defined secretion sites in the cell wall peptidoglycan of gram-positive bacteria.

Peptidoglycan Turnover or Catalyzed Release of Cell Wall-Anchored Surface Proteins?

Pancholi and Fischetti observed that streptococci pretreated with phage lysin amidase would release large amounts of M protein (612). Phage lysin treatment is performed at pH 5.5 for optimal enzymatic activity, and during this treatment relatively little surface protein is released. However, once the pH of the buffer containing predigested streptococci was adjusted to pH 7.0 or higher, large amounts of M protein were released into the medium. The authors investigated the enzymatic activity that releases M proteins into the medium, assuming that it would cleave a membrane anchor of M proteins. The releasing activity is inhibited by pHMB, zinc, or other divalent cations. In view of the sorting hypothesis described above, it is conceivable that the measured solubilization is that of cell wall-linked

M proteins. A change in the pH of intact streptococci does not lead to the release of M proteins.

The release of surface protein upon binding of specific antibody has been investigated for *S. mutans* P1 protein (476a–476c). Incubation at pH 6 caused the release of the surface protein antigen with its bound antibody from the streptococci (476a, 476b). The biological relevance of this was tested in an experiment that measured the detachment of *S. mutans* cells from a biofilm, which occurred only under conditions that also permitted the release of surface protein (476c). Shedding of P1 antigen required live cells, suggesting a pathogenic mechanism conceptually similar to another established paradigm, the shedding of variable surface antigen by trypanosome species (227). These organisms anchor their surface antigen via a GPI moiety to the cytoplasmic membrane, and cleavage with a phospholipase C allows the release of these peptides into the surrounding medium upon incubation with cross-linking antibodies. This phenomenon is thought to be one essential aspect of a complex mechanism by which trypanosomes establish antigenic variation of their surface protein antigen.

Cell Wall-Spanning Segments of Surface Proteins

The domain of protein A that is protected from added protease by the staphylococcal cell wall (region X) has a remarkable repeat structure and is about 175 residues long (291). The fact that thermolysin digestion generated a uniformly protected C-terminal peptide suggests that the distance between the cell wall-anchoring point and the surface-displayed portion is similar for all protein A molecules. Hence, protein A molecules that are linked to the cell wall do not "migrate" toward the cell surface, as would be implied by a model in which the cell wall is synthesized from the inside out, like the bark of an aging tree. Rather, all protein A molecules are linked to the cell wall and remain at that distance as the cell wall grows longitudinally over the growing cytoplasmic membrane. If this is so, the distance of the wall-spanning segment between the anchoring and the folded portions of surface protein should be critical in determining the functionality of these molecules. This was tested for *S. aureus* fibronectin-binding protein (FnBPB) expressed in *Staphylococcus carnosus*, and it was found that decreasing amounts of wall-spanning region caused reduced surface display of a fused lipase or β-lactamase domain (757). Furthermore, if the wall-spanning segment of FnBPB was shorter than 90 amino acids, the activity of the recombinant enzymes was reduced or abolished, presumably because the peptidoglycan exoskeleton interfered with the folding of the fused lipase or β-lactamase domains.

Other surface proteins harbor wall-spanning segments that also display a repeat structure, albeit with differences in sequence. Protease protection experiments of M proteins have yielded similar results to those described for protein A above (613). The wall-spanning domain of clumping factor (ClfA) of *S. aureus* consists of tandem repeats of an aspartyl-serine dipeptide. This domain functions to present the fibrinogen binding domain on the staphylococcal surface, and successive truncations of the repeat domain cause reductions in surface display (308).

ENGINEERED DISPLAY OF PROTEINS ON THE SURFACE OF GRAM-POSITIVE BACTERIA

Many gram-positive species colonize the mucosal surfaces of humans or animals without ever causing disease. These species constantly interact with the host immune system, which prevents their penetration into deeper tissues and establishment of disease. Although the cellular immune system and the phagocytosis of bacteria are critical in defending the host mucosal surfaces, it is thought that the humoral immune system also contributes to this defense via secretory IgA molecules (581). Other, more pathogenic microorganisms have the ability to penetrate mucosal surfaces and multiply within the host. On a first encounter with such microbes, the host may be defenseless, and the outcome of this interaction might result in disease. Pozzi and coworkers developed the gram-positive commensal organism *Streptococcus gordonii* as a delivery system to generate a mucosal immune response against such pathogens (215, 649, 650). Surface display of epitopes or entire proteins requires an N-terminal signal peptide and a C-terminal sorting signal for cell wall anchoring (716). For increased stability and surface display, several vectors that allow fusions with the scaffold of surface proteins of gram-positive bacteria are available (593). Inoculation of these engineered *S. gordonii* strains into naive animals led to colonization of mucosal surfaces and to the generation of a mucosal immune response (158, 540–542). Similar strategies for the immunization of mucosal surfaces have been established for *Staphylococcus xylosus* and *Lactococcus lactis*, two other nonpathogenic commensal species (583, 631, 752).

Surface display of engineered proteins might also be useful for biotechnological purposes. For example, recombinant antibodies are needed for diagnostic and therapeutic approaches. To facilitate their selection, these antibodies are often displayed on the surface of filamentous phages or on the surface of *E. coli* (518). Certain limitations apply, because these antibodies have to fit within the scaffold of phage proteins that are incorporated into an infectious particle. Several laboratories have used staphylococci as hosts for the expression of recombinant surface proteins. Engineered *S. carnosus* and *S. xylosus* were shown to display IgG as well as several other polypeptides on the cell surface (288, 582, 583, 697, 757). Surface-displayed antibodies may be manipulated by genetic or chemical means, and this technology may provide some advantage over phage display.

CELL WALL-ANCHORED SURFACE PROTEINS IN GRAM-POSITIVE BACTERIA

Surface proteins are displayed on the cell surface in order to interact with a substrate located in the surrounding environment. These proteins can have a wide range of functions including binding of host tissues, binding to specific immune system components, protein processing, nutrient acquisition, and interbacterial aggregation for the conjugal transfer of DNA. This section briefly reviews the various proteins identified thus far that possess C-terminal cell wall sorting signals. It is intended to provide a reference for those who are interested in the functions of various surface proteins and to highlight the incredible functional diversity found in this class of proteins. It is important to note that covalent attachment of proteins to the peptidoglycan has been conclusively demonstrated only for a few of the proteins reviewed below. Future studies should determine conclusively whether cell wall sorting is truly a universal process among gram-positive bacteria. It should be noted, however, that no proteins containing cell wall-sorting signals have been identified in the spore-forming gram-positive genera such as *Bacillus* and *Clostridium*.

Common Features of Anchored Surface Proteins

Anchored surface proteins must span the thick cell wall of gram-positive bacteria in order to display their functional do-

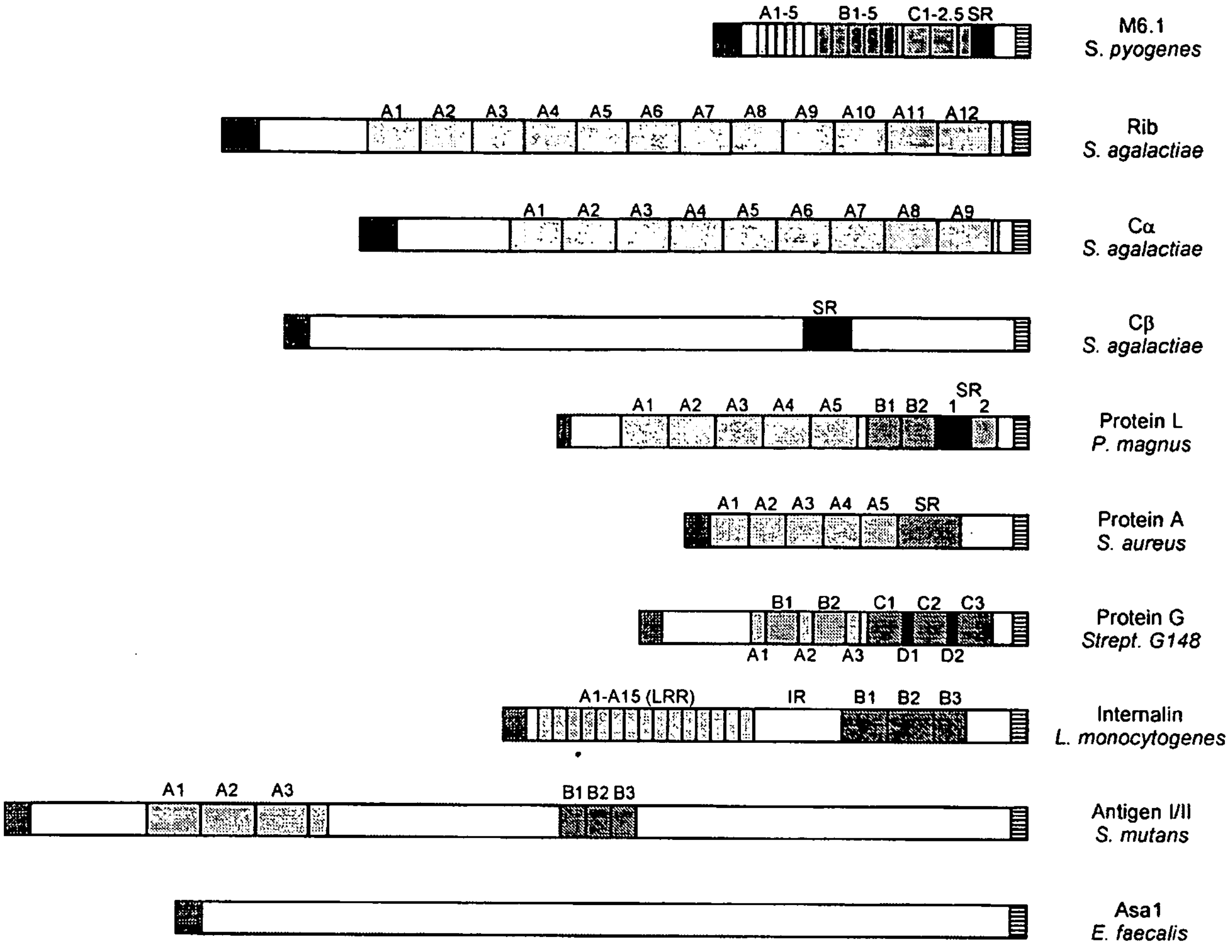


FIG. 15. Diagram of the structures of several anchored surface proteins from various gram-positive bacteria. All surface proteins possess an N-terminal leader peptide (N) and a C-terminal cell wall sorting signal. Many surface proteins possess several repeating domains. Particularly striking are the Cα and Rib proteins of *S. agalactiae*, which are composed of 9 or 12 nearly identical repeat domains, respectively (see the text). References for these proteins are given in Table 1.

mains to the surrounding environment. Despite their diverse range of functions, several common themes in the design of cell wall-anchored proteins of gram-positive bacteria are apparent (Fig. 15). N-terminal domains that contain the binding or catalytic activities are frequently followed by a set of repeat domains that may or may not possess activity. Often there is also a proline-rich stretch of amino acids immediately preceding the LPXTG motif that is thought to introduce random coils in the structure to assist in traversing the peptidoglycan network (212). It is possible, however, that such a domain has another function, since the proline-rich domains of several proteins are found to be more N-terminal than are regions predicted to span the cell wall.

Many cell wall-sorted surface proteins contain a number of tandem repeat domains that can vary in size from a few to several hundred amino acids. Striking examples are the alpha and Rib proteins of group B streptococci, which possess 9 or 13 repeats identical in both amino acid and nucleotide sequence (837). Strain-to-strain variation in the exact number of tandem repeat domains has been observed for many surface proteins, indicating that these domains are targets for recombination and/or duplication. Several mechanisms are likely to be involved in the generation of these repeat domains (139, 419, 420).

Functions of Cell Wall-Anchored Surface Proteins

Binding of immunoglobulins. Much is known about the Ig binding surface proteins of gram-positive bacteria. Since the discovery of staphylococcal protein A, several other Ig binding proteins have been characterized and many of their genes have been cloned and sequenced. In 1977, Myhre and Kronvall proposed three different classes of Fc receptors based on the ability of certain bacteria to bind several different Ig subtypes from various mammalian species (569). Types I and II were the designations given to staphylococcal protein A and the streptococcal Fc receptors (M proteins) respectively. Type III was the designation given the Fc binding protein of group C and G streptococci (protein G). These designations were established before the genes encoding any of the Ig binding proteins had been determined; however, this nomenclature is still widely used today. For proteins A, G, and L, the three-dimensional structures of the antibody binding domains have been determined (143, 286, 854). With the exception of protein L, all surface protein Ig receptors bind to the heavy-chain portion of the antibody at the interface between the Fc C_H2 and C_H3 domains (231). Remarkably, despite their similar binding specificity, proteins A and G and the streptococcal Fc receptors (M proteins) have no structural similarities and appear to be the result of convergent evolution (231). Although several gram-positive bacterial species possess IgG binding activity, only two, the group A and B streptococci, have demonstrated the ability to bind IgA. Similar to the situation for the IgG receptors, the IgA receptors of GBS and GAS are unrelated to each other and are also most probably the result of convergent evolution.

Binding of serum and ECM proteins (MSCRAMMs). Adherence to host tissues is the first step in the establishment of an infection. Many pathogenic gram-positive bacteria do not express structures such as pili and instead utilize cell wall-anchored surface proteins to adhere either to the extracellular matrix (ECM) or to other components present in host tissues. Höök and colleagues have given the name MSCRAMMs (microbial surface components recognizing adhesive matrix molecules) to surface proteins that bind to components of the ECM (621, 624). MSCRAMMs have been found in almost all pathogenic gram-positive species, and their modular design and common binding domains suggests that they have arisen from a series of recombinational events and horizontal gene transfer. Many MSCRAMMs are capable of binding to more than one ECM component, and a single strain often possesses several different proteins that bind the same host component. For example, *S. pyogenes* has at least three proteins capable of binding fibronectin while *Streptococcus dysgalactiae* and some *S. aureus* strains have two (see below). MSCRAMMs and other adhesive surface proteins are the subject of a number of reviews (621, 624, 844).

Other surface proteins bind a wide variety of serum proteins including albumin, complement regulatory factors, soluble forms of fibronectin and fibrinogen, and the proinflammatory molecules plasmin(ogen) and kininogen. In addition, several bacterial surface proteins bind molecules present on the surface of host cells, a function that may help the bacteria either adhere or invade. Surface proteins that bind serum molecules or molecules found on the cell surface are not technically defined as MSCRAMMs even though these molecules may play a role in adherence to host tissues (624).

Wall-anchored enzymes. Several examples have emerged where a metabolic enzyme is surface attached, particularly in cases where the function of the enzyme is to break down a large, nontransportable nutrient polymer into smaller subunits that can subsequently be taken up into the cell by a permease system. This is the case for both the dextranases and fructosidases of the mutans streptococci and the casein peptidase of lactococci. Not all surface-localized enzymes perform a nutrient acquisition function for the cell, however. For example, the nisin leader peptidase is involved in the processing of the nisin precursor to an active form by the removal of its leader peptide. Another surface protease, the streptococcal C5a peptidase, is involved in the degradation of this chemoattractant, presumably to prevent the recruitment of polymorphonuclear neutrophils (PMNs) and macrophages to the site of infection (see below).

Bacterial aggregation. Both the conjugal transfer of DNA and the formation of dental plaque require that bacterial cells be able to adhere to one another through the specific interactions of surface proteins (160, 168, 441). In a majority of cases, the gram-positive bacteria employ cell wall-anchored surface proteins for this purpose. A notable exception is the fimbriae of the actinomycetes, whose subunits surprisingly contain C-terminal cell wall sorting signals (see below).

Streptococcus pyogenes (Group A Streptococci)

The work of Rebecca Lancefield, which began over 70 years ago, demonstrated that streptococcal surface proteins are mediators of virulence as well as targets for acquired immunity (467). Early studies determined that the surface proteins of GAS displayed a high degree of antigenic diversity. These findings led to the currently used typing system for GAS that is based on antisera raised against a set of variable surface antigens designated M, T, and R (212, 468). Of these, the M antigens are the most serologically diverse, with over 80 different serotypes identified thus far (see below). Considerable effort has been put into determining the relationship between the antigenic profile of a strain of GAS and its ability to cause disease (213, 420).

The GAS are also subclassified by whether they express the serum opacity factor (OF), an apoproteinase whose expression causes serum to become opaque (see below). Approximately half of streptococcal isolates express OF, and this expression pattern generally correlates with certain M serotypes. For example, M types 2, 4, 8, 9, 13, 22, 49, 59, 62, and 76 are often found to be OF^+ whereas M types 1, 3, 5, 6, 12, 14, 18, 19, 24, 55, 57, and 80 are usually OF^-. The M41 strain D421 is OF^+, whereas another M41 strain, D463, is OF^-, demonstrating that exceptions to this general rule exist (658). A table compiling the various M, T, and OF types has been published (389), and streptococcal surface proteins and typing systems have been the subject of a number of excellent past reviews (212, 419, 467). Therefore, this section is focused primarily on findings that have been made in the past decade.

It has recently become possible to analyze the genetic elements responsible for the antigenic diversity displayed by GAS at the nucleotide level. Until a decade ago, it was assumed that the antigenic properties of the various M types were due to the expression of a single variable M antigen. However, it is now evident that many strains of GAS, including almost all OF^+ isolates, express more than one member of the M-protein family (295, 607, 632, 753) and that there can be considerable genetic diversity within a single M serotype (152, 627). In addition, there are many cases where M proteins from strains considered to be of a different M serotype have identical N termini (193, 848), suggesting that some type-specific antisera may be directed against more than one of the expressed M proteins (see below). Such findings have led many to reassess the previous assumptions that stemmed from early serological studies.

Mga regulon. The genes encoding the OF, the C5a peptidase, and M proteins are all regulated at least in part by a multiple gene regulator called Mga (formerly *virR* or *mry* [111, 718, 729]) (117, 470, 634, 639). The *mga* gene encodes a 62-kDa DNA binding protein that activates transcription by binding to a consensus 45-nucleotide binding site found in the promoters of the regulon members (533, 536). Mga also positively regulates the expression of the genes encoding a secreted complement inhibitor (*sic*) (9, 427), streptococcin A (*scnA*), a cysteine protease (*speB*), and an oligopeptide permease system (*opp*) while down-regulating the expression of streptolysin S (639). There is some evidence, however, that other elements are involved in the regulation of these genes, including transcriptional terminators that serve to down-regulate the expression of the *scpA* gene and a neighboring open reading frame (71, 637, 639, 652). Mga bears some homology to the effector proteins of bacterial two-component regulatory systems, although a cognate sensor-kinase for this protein has not been identified. The expression of Mga and surface proteins in GAS is regulated by a variety of environmental conditions (534) including elevated levels of carbon dioxide (110, 595, 637) and/or the growth phase (535).

Mga regulons are ubiquitous throughout GAS (633), but their exact organization varies from strain to strain, particularly in the number and types of M-protein genes present (295, 296, 344, 632). In all cases, the *mga* gene is located immediately upstream of the M-protein genes (*mrp*, *emm*, or *enn*) and the gene encoding the C5a peptidase (*scpA*).

Streptococcal M-protein family (Emm, Enn, and Mrp). The M family of streptococcal surface proteins is composed of the related Emm (class I and II), Mrp (FcrA), and Enn proteins. The Mga regulons of OF^+ strains of GAS always contain all three M-family proteins in the order *mrp*, *emm*, and *enn* followed by the *scpA* gene. In contrast, the organization of the Mga regulons in OF^- strains is much more variable and may include either one, two, or all three of the M-family genes, although the *emm* gene is always found (344). In at least one case, an additional open reading frame encoding a surface protein of unknown function (*orfX*) controlled by Mga has been found downstream of *scpA* (639).

Members of the M family of proteins are elongated dimeric alpha-helical coiled-coil molecules that can bind a variety of host components including Igs (5, 7, 75, 229, 320), fibrinogen (358, 409, 830, 850), kininogens (35), plasminogen (42), and albumin (229, 669) as well as factors that inhibit the deposition of complement on the bacterial surface (354, 780). A source of some confusion is that different laboratories have assigned different names to similar M-family proteins (e.g., the various class II Emm proteins have been designated Arp, Sir, or Emm). To assist the reader, the different names and ligands of several M-protein family members that have been sequenced are summarized in Table 2.

The designation "M protein" has traditionally been reserved for members of the M family that are known to possess antiphagocytic properties, whereas all similar proteins with unknown or no antiphagocytic properties are generally designated "M-like." Recent studies, however, have blurred this distinction, and it has become necessary to reassess the classical definition of an M protein. Separate laboratories have found that both the Mrp and Emm proteins can have antiphagocytic properties in a given strain (638, 781). It is also unclear whether M proteins play an equal role in protection from phagocytosis in every strain (137, 496) or whether a given M protein will have antiphagocytic properties when expressed in all strains (361, 781). Also, recent findings suggest that heparin, added as a blood anticoagulant in most phagocytosis assays, may interfere with the function of some M proteins by competing against the binding of factor H (62, 445, 781) (see below). If this is true, the antiphagocytic function of proteins previously considered to be M-like may have to be reassessed. For these reasons, we choose to adopt the functionally neutral terms Mrp, Emm, and Enn used by other laboratories to identify individual proteins based on their specific structural features while using the term "M protein" to designate all members of the family irrespective of their antiphagocytic function (345, 635, 781, 848). The mechanisms by which M proteins are thought to prevent phagocytosis are discussed below.

Sequencing of the various M proteins has revealed several common features in their molecular design, which are summarized in Fig. 16. The N-terminal hypervariable domains of mature M proteins account for the observed antigenic diversity of these molecules. In contrast, the central and C-terminal domains are almost completely conserved within each specific M subfamily (420). Throughout almost the entire M-protein sequence are heptad repeat motifs whose first and fourth amino acids are apolar. This arrangement is typical of coiled-coil molecules and serves to display the hydrophobic amino acids on the face of an alpha helix serving as a surface for dimerization (212). The heptad repeats are found even in the highly variable N-terminal domains, although not always in an optimal distribution, indicating that there is considerable selective pressure to retain the coiled-coil conformations (212, 217, 515, 586).

All members of the M family possess an N-terminal leader peptide and a typical C-terminal cell wall sorting signal. The cell wall localization of M and M-like proteins was determined as early as 1952 (27, 228, 696), and it was demonstrated that purified cell walls could be used as a source for M protein (410, 446). Covalent attachment to the cell wall has been demonstrated by the fact that M protein solubilized from the cell wall with phage-associated lysin migrates as a ladder of bands on SDS-PAGE. More recently, it has been shown that the M6 protein is cell wall anchored when expressed in the gram-positive lactic acid bacteria (631).

Emm proteins (class I and class II). The class I Emm proteins are found almost exclusively in OF^- GAS. They were originally distinguished from the class II Emm proteins on the basis of their reactivity with a set of monoclonal antibodies raised against their C-terminal repeats (49). The binding properties of the class I Emm proteins are remarkably diverse (Table 2), but most have been shown to bind fibrinogen and/or albumin. These binding functions appear to have been divided between the Emm (class II) and Mrp proteins in the OF^+ strains of GAS (see below).

The class II Emm proteins are typically found in GAS of the OF^+ lineage and are the molecules usually recognized by M-typing antisera. These proteins were initially distinguished from their class I counterparts due to their inability to react with certain monoclonal antibodies directed against the C repeats of class I Emm molecules. None of the class II Emm proteins have been found to bind fibrinogen, a function that seems to be compensated for by the Mrp proteins in the OF^+ strains. In addition, all class II Emm proteins studied thus far bind Ig, although their exact binding preferences differ (see Table 2).

Mrp (FcrA). Mrp proteins are usually found in GAS strains of the OF^+ lineage and are always encoded immediately downstream of the *mga* gene (Fig. 16). All Mrp proteins characterized thus far bind both human IgG (subclasses 1, 2, and 4) and fibrinogen (753). They can be distinguished from the Emm and Enn proteins by the absence of the conserved C-repeat domains. Instead, these proteins possess two or more copies of a different type of central repeat designated "A repeats" (295, 607), in which the IgG binding properties are thought to reside (8, 312). Upstream of the conserved repeats in both the Mrp and class II Emm proteins is a domain of unknown function that is rich in glutamate and glutamine (EQ domains). Unlike in the Emm proteins, the N-terminal domains of the Mrp proteins are not highly variable. Sequencing of the N-terminal regions of Mrp proteins from 37 different M serotypes revealed the existence of only six different types of N-terminal Mrp domains (447). In at least four cases (Mrp2, Mrp22, Mrp49, and Mrp64/14), an Mrp protein has shown evidence of being antiphagocytic (638, 781), and this property may be due to their ability to bind fibrinogen (see below).

Enn. The genes encoding the Enn proteins are found almost exclusively in OF^+ strains as the third open reading frame following *mga* in the Mga regulon (Fig. 16). Instead of the EQ-rich domains found in the class II Emm and Mrp proteins, the "C-repeat" domain of Enn proteins are usually preceded by a conserved set of amino acids (EDLKTTLAKTTKEN). The Enn proteins, like the Mrp proteins, do not display the same high degree of variability displayed by the Emm proteins (847). Recombinant Enn proteins expressed in *E. coli* are generally found to have IgA binding activity (51).

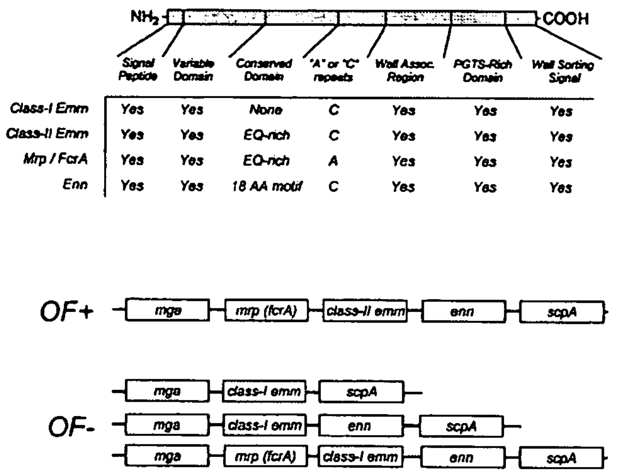
Thus far, no members of the Enn family have been demon-

TABLE 2. M proteins and their ligands

M protein	Strain	Reference(s)	M type	Experimentally demonstrated ligands
Class I Emm				
M1 (Emm1)	AP1	8	1	Albumin (712), fibrinogen (8, 712), IgG (712), kininogen (33)
M1.0 (Emm1.0)	CS130	297, 306	1	
M1.1 (Emm1.1)	CS190	306	1	
M1.2 (Emm1.2)	T1/29/58	306	1	
M3 (Emm3)	4/55	416, 666	3	Albumin (666), fibrinogen (666), fibronectin (666)
M5 (Emm5)	Manfredo	552	5	Fibrinogen (851), factor H (445), FHL-1 (445)
M5.8193	NCTC 8193	849	5	Fibrinogen (849)
M6 (Emm6.1)	D471	346	6	CD46 (596), factor H (354), FHL-1 (445), fibrinogen (851), kininogen (33)
M12 (Emm12)	CS24	671	12	Albumin (666), IgG (666)
M18.1 (Emm18.1)	87-282	137	18	
M24 (Emm24)	Vaughn	560	24	Fibrinogen (850)
M41 (Emm41)	71-710	632	41	Plasminogen (112)
M52 (Emm52)	71-719	632	52	Plasminogen (112)
EmmL55 (Emm55)	A928	77	55	Albumin (77), fibrinogen (77), IgG (77)
M57 (Emm57)	A995	515	57	
Class II Emm				
Emm2 (EmmL2.1)	T2/MR	51	2	
Emm4 (Arp4)	AP4	232	4	C4BP (780), IgA (5), IgG (753)
Emm9	71-683	635	9	IgG (635)
EmmL15 (Emm15)	EF1949	415	15	
Emm22 (Sir22)	AL168	754	22	C4BP (780), IgA (753), IgG (753)
Emm28 (Sir28)	AL368	488, 753	28	IgA (753), IgG (753)
Emm49 (EmmL49)	CS101	295	49	Albumin (229), IgG (229)
Emm50 (EmmL50)	B514	877	50	IgG (877)
Emm53 (PAM)	M53	42	53	Plasminogen (42, 112)
Emm60 (Arp60)	AW43	320	60	C4BP (780), IgA (320), IgG (486)
Emm64/14	64/14	75	NT	IgG (75)
Mrp				
Mrp4	AP4	607	4	Fibrinogen (607), IgG (607)
Mrp8	4025	447	8	Fibrinogen (447), IgG (447)
Mrp9	71-683	447	9	Fibrinogen (447), IgG (447)
Mrp13	71-686	447	13	Fibrinogen (447), IgG (447)
Mrp15	EF1949	415	15	
Mrp22	AL168	754	22	Fibrinogen (753), IgG (753)
Mrp28	AL368	753	28	Fibrinogen (753), IgG (753)
Mrp49 (FcrA49)	CS101	636	49	Fibrinogen (447), IgG (636)
Mrp50	B514	877	50	IgG (877)
Mrp60	AW43	753	60	Fibrinogen (753), IgG (753)
Mrp76 (FcrA76)	CS110	313	76	Fibrinogen (607), IgG (607)
Mrp64/14 (Fcr64/14)	64/14	75	NT	Fibrinogen (447), IgG (75, 447)
Enn (Emm IIb)				
Enn2 (EmmL2.2)	T2/MR	51	2	IgA (51)
Enn4	AP4	381	4	IgA (391)
Enn15	EF1949	415	15	
Enn22	AL168	754	22	
Enn49 (EnnX)	CS101	295	49	
Enn50	B514	877	50	IgA (877)
Mosaic Enn				
Protein H	AP1	273	1	Albumin (229), C4BP (393), IgG (7), fibronectin (230), MHC-II (8), N-CAM (230)
Enn5.8193	NCTC8193	849	5	IgG (849)
Enn64/14	64/14	635	NT	IgG3 (635)

strated to have antiphagocytic properties. Under laboratory conditions, it has been demonstrated that the expression of *enn* genes is consistently more than 30-fold lower than the expression levels found for both *mrp* and *emm* (51, 381, 496, 849, 877). The low expression level has led to the hypothesis that the *enn* genes act primarily as a "sequence reservoir" for recombination with the *emm* and *mrp* genes. Indeed some unusual "mosaic" Enn proteins including protein H, Enn 5.8193, and Enn 64/14 all appear to have been generated by recombination between an *emm* gene and an *enn* gene (see below). Low expression of Enn has not been demonstrated during in situ, however, and it is possible that the Enn proteins do play a role during an infection.

Binding properties of M proteins: role in antiphagocytosis, adhesion, inflammation, and invasion. It was demonstrated long ago that GAS strains are resistant to phagocytosis in



	Signal Peptide	Variable Domain	Conserved Domain	"A" or "C" repeats	Wall Assoc. Region	PGTS-Rich Domain	Wall Sorting Signal
Class-I Emm	Yes	Yes	None	C	Yes	Yes	Yes
Class-II Emm	Yes	Yes	EQ-rich	C	Yes	Yes	Yes
Mrp / FcrA	Yes	Yes	EQ-rich	A	Yes	Yes	Yes
Enn	Yes	Yes	18 AA motif	C	Yes	Yes	Yes

FIG. 16. Domain structure of the M-protein family and the structure of the Mga regulons from OF$^+$ and OF$^-$ strains of GAS (see the text).

human blood in the absence of specific antibodies directed against the M proteins (467). In 1979, it was demonstrated that M-protein-deficient strains of GAS could be efficiently opsonized and cleared by the alternative complement pathway (55, 630). The alternative complement pathway ultimately results in the deposition of C3b on the surface of the bacteria, which can subsequently act as a receptor for phagocytic immune system cells. Jacks-Weis and colleagues found that strains of GAS expressing M protein accumulate approximately fourfold less C3b on their surface than did mutant strains (374). Several laboratories have since attempted to elucidate the molecular basis of the antiphagocytic properties of the M proteins and their possible effect on the alternative complement pathway. These studies have variously attributed the antiphagocytic property of the M proteins to their ability to bind fibrinogen (355), factor H (62, 214, 354), FHL-1, C4BP (392, 393, 780), or C1q (443).

Factor H, FHL-1, and C4BP belong to the RCA (for "regulators of complement activation") family of molecules that are capable of regulating complement activity. The proteins in this family are composed of multiple, highly homologous domains known as short consensus repeats (SCRs) (356). SCRs domains are each composed of approximately 60 amino acids, of which 4 cysteines and a few other residues are conserved and provide the structural framework for the domain while the remaining amino acids are thought to contribute to the binding specificity. SCRs, like many other common protein domains, are modular and are believed to act independently of other domains in a protein (28). Factor H is a soluble molecule composed of 20 SCRs, which compose almost the entire protein (356). FHL-1 (for "factor H-like") is a truncated variant of factor H generated by alternate mRNA splicing and is composed of the first seven SCR domains of factor H (356, 553). C4BP is a soluble regulator of complement that is composed of seven alpha chains and one beta chain, which contain eight and three SCRs each, respectively (356).

The finding that an M protein could bind a down-regulator of the alternative complement pathway was first demonstrated for factor H (354). The binding of factor H to the class I M6 protein was mapped to the C-repeat region (214); however, this finding has not been without some controversy. For example, the C-repeat domains was shown to bind albumin by another laboratory (8, 229, 230), although the possibility exists that the C repeats can bind multiple ligands. In addition, fibrinogen was found to compete for factor H binding to the M6 protein even though fibrinogen binding is believed to occur in the B repeats of M6, upstream of the C repeats (355). It was suggested in that study, however, that fibrinogen could form a complex with factor H and that this fibrinogen-factor H complex would be responsible for providing protection from phagocytosis (355). This hypothesis may explain the previously observed antiphagocytic properties of fibrinogen. One study of a GAS strain (JRS251) expressing an M6 protein lacking the C repeats indicated that this strain was no longer able to bind factor H even though it was still resistant to phagocytosis (628). A more recent study, however, has demonstrated that these mutant cells can bind factor H, albeit at lower levels (724). Given this data, it appears unlikely that the binding site of factor H lies within the C-repeat domain.

Recent findings have suggested that the physiologically relevant binding partner of the class I Emm proteins is not factor H but, rather, FHL-1 (392, 445). Surprisingly, the binding of FHL-1 occurs through the hypervariable N-terminal domain of the M protein. Three separate laboratories have identified a domain found in both factor H and FHL-1 (SCR7) as being responsible for interacting with the M proteins (62, 445, 724). Given this data, it is difficult to envision how the N-terminal domains would be capable of selectively binding FHL-1 over factor H. It has been noted, however, that the properties of FHL-1 and factor H differ both structurally and functionally (326). In addition, it has been demonstrated that the binding of radiolabeled FHL-1 to the M5 protein can be inhibited only weakly by factor H and not at all by fibrinogen and that FHL-1 bound to bacteria retains its complement inhibitory function (392). Also, the binding of FHL-1 appears to occur under physiologically relevant conditions whereas the binding of factor H is inhibited by fibrinogen at the concentrations present in plasma (355, 392). This data suggests that there are two factor H binding sites on the M5 protein, one of which binds factor H (see above) while the other has a greater affinity for FHL-1 and lies outside of the fibrinogen binding and C-repeat regions. Such a model is in agreement with other observations made by Sharma and Pangburn (724) and would explain why M proteins lacking C repeats retain antiphagocytic activity (628). It should be noted that the characterization of the N-terminal FHL-1 binding domain was carried out by fusing domains onto an M protein without FHL-1 binding activity. This type of "gain-of-function" binding study avoids certain experimental concerns raised by the negative data obtained from binding studies that employ deletion mutants, such as the possibility of deleterious effects caused by conformational changes.

Several Emm proteins, particularly those in the class II subfamily, do not have the ability to bind either factor H or FHL-1. Instead, it has been demonstrated that many of these proteins bind C4BP. Similar to the binding studies of FHL-1, it was determined that the binding of C4BP occurs through the hypervariable N-terminal domain (393). The FHL-1 and C4BP binding data suggest a model whereby the N-terminal hypervariable domains of M proteins have evolved the ability to bind complement regulatory molecules (392). Interestingly, no consensus amino acid motif has been identified in the hypervariable regions demonstrated to bind C4BP (393). This data suggests that the N-terminal domains of the M proteins arise from two opposing selective pressures, the ability to maintain their antiphagocytic function while simultaneously altering their antigenic structure (393). This model may explain why the N-terminal variable domain is so critical to the function of M proteins and has not been lost due to selective pressure. It would also explain why mutant M6 proteins lacking C-repeat domains retain their antiphagocytic function.

Very recently, C1q, another complement component, was suggested to bind to the M proteins FcrA76 (Mrp76) and M5 (443). The binding studies were carried out on crude acid extracts, and the identification of the two M proteins was based solely on their apparent masses on SDS-PAGE. More studies are therefore necessary to demonstrate if this binding actually occurs through the M proteins and is biologically relevant in vivo.

At this point it should be mentioned that there is still some debate about whether M proteins are either necessary or sufficient for protection from phagocytosis in all GAS strains. For example, a mutant strain that lacks a hyaluronic acid capsule but expresses wild-type levels of M protein is sensitive to phagocytosis whereas the parent strain (strain 87-282) is resistant (841). In a later study, it was shown that strain Vaughn (M type 24) required fibrinogen for optimal resistance to opsonization and phagocytosis whereas in surprising contrast, strain 87-282 (M type 18) was opsonized by C3b in either the presence or absence of fibrinogen but in both cases remained resistant to phagocytosis (137). These results suggest that in some strains the deposition of C3b does not necessarily lead to phagocytic killing. It has recently been reported that a mutant of 87-282, in which the M protein was insertionally inactivated, remained resistant to phagocytotic killing in blood (496). The 87-282 mutant strain used in these studies still had an intact *enn* gene, although the Enn expression levels appeared to be quite low. The Emm18.1 protein from this strain was demonstrated to confer protection from phagocytosis to a strain of a different M type, indicating that the M protein from strain 87-282 is indeed functional (134). Nevertheless, these data suggest that the hyaluronic acid capsule can, by itself, protect the bacteria from phagocytosis in strains like 87-282 whereas other strains also require the expression of an M protein.

The ability of many different M proteins to bind Ig has been clearly demonstrated. The IgA binding site of Arp4 (Emm4) has been mapped by separate laboratories to a short stretch of residues near the N terminus (50, 391). A consensus binding site was identified and found to be present in most known IgA binding M proteins including Arp60, Enn4, Enn2.2, Enn50, and Sir22. Binding to IgG has also been clearly demonstrated, but the situation is much less clear. It is apparent that some M proteins bind human IgG1, IgG2, and IgG4 whereas others bind only IgG3 and a few M proteins have the ability to bind all four subclasses (summarized in reference 75). This leads to the tempting speculation that two or more different binding sites exist on M proteins, one of which binds IgG1, IgG2, and IgG4 while the other binds IgG3. Indeed, Björck and colleagues have found two separate IgG binding sites on protein H and M1. Domains homologous to the protein H IgG binding site were also found in the A repeats of the Mrp proteins, suggesting that such domains may bind IgG1, IgG2, and IgG4 (229). The IgG binding site of M1 is homologous to a region found in Arp4, a protein that can bind only a limited subset of IgG molecules, including IgG3 but not IgG1, IgG2, or IgG4 (8). Despite this correlation, the protein H and M1 IgG binding domains are found only in a subset of the Emm proteins known to have IgG binding activity. Obviously, more experiments must be performed to determine the locations of the various IgG binding domains.

As mentioned above, surface proteins can be released from some gram-positive cells by the action of specific proteases. In GAS, biologically active fragments of protein H, M1, and C5a peptidase can all be released from the cell surface by the action of SCP, a streptococcal cysteine protease (39). It has recently been shown that a soluble protein H-antibody complex can mediate the activation of the classical (antibody-mediated) pathway of complement (40). When immobilized on a surface, however, the protein H-antibody complex was found to instead inhibit the activation of complement. These findings suggest that the role of the Ig binding domains may involve inhibition of the classical complement-mediated pathway at the surface of the bacteria while the soluble forms of these proteins may serve to deplete complement at sites further away from the bacteria (40).

M proteins have been suggested to play a role in the generation of an inflammatory response by the binding of fibrinogen (831), kininogen (34), or plasminogen (76). By doing so, the bacteria may gain the ability to invade deeper tissues by increasing vascular permeability, dissolving clots, and disrupting the tight interactions within the ECM. Björck and colleagues tested the kininogen binding abilities of 49 strains of GAS and found that 41 were able to bind radiolabeled kininogen to their surface (35). This binding ability correlated well with the presence of M protein, since none of six M-protein-deficient strains were capable of binding kininogens. Direct analysis of kininogen binding to three M proteins revealed that the interaction occurs within the N-terminal portion of the M protein at a site that does not overlap the albumin, fibrinogen, or IgG binding sites (35).

Plasminogen is the biologically inactive precursor to plasmin, a fibrinolytic molecule speculated to aid in the invasive properties of cells (76). The role of plasmin(ogen) binding on the surface of GAS is not entirely clear but is apparently of some importance since more than 10 different species of invasive bacterial pathogens have been shown to bind these molecules (76). Most strains of GAS possess a surface-exposed glyceraldehyde-3-dehydrogenase-like molecule (SDH) that binds plasmin and not plasminogen (614). In addition, five GAS strains, of M serotypes 33, 41, 52, 53, and 56, that are usually associated with skin infections possess M proteins with plasminogen binding activity (42, 112). The common plasminogen binding motif in these M proteins was identified and localized to a set of tandem repeats found within the N-terminal domain (112). Surprisingly, Pancholi and Fischetti have recently reported the presence of a surface-localized alpha-enolase (SEN) that binds both plasmin and plasminogen (610). The finding that SEN is expressed ubiquitously throughout all strains of GAS conflicts with the finding that surface binding of plasminogen is a rare occurrence (112, 610). The reason for this discrepancy is unclear. Both SEN and SDH belong to a recently identified group of unusual enzymes that have the ability to bind plasma components. Their role in virulence and even their surface localization are under considerable debate, since they do not possess N-terminal leader peptides. These proteins are discussed further below.

Like plasminogen, kininogen is a biologically inactive precursor molecule. Proteolytic activation of kininogen generates the peptide hormone bradykinin, a vasoactive molecule that may assist in the generation of a localized inflammatory response near the site of infection. The effect of M-protein binding to either plasminogen or kininogen does not interfere with the ability of either molecule to be proteolytically converted into a biologically active form by their respective cognate proteases (34, 112).

It has long been speculated that M proteins may also act as adhesins to host tissue, but the mechanisms by which this may occur remain unclear (180), although adhesion has recently been implicated in the ability of GAS to generate a proinflammatory response in keratinocytes (829). Expression of the Emm5, Emm18, and Emm24 proteins in a deletion mutant of strain Vaughn enables these cells to adhere to HEp-2 cells. The M6 protein has been shown to mediate adherence to both HEp-2 cells (832) and keratinocytes (597). The binding of M6-expressing cells to HEp-2 cells appears to require fucose-containing glycoproteins (833). On the other hand, the ligand on the surface of keratinocytes responsible for binding M proteins was identified as the membrane cofactor protein (MCP or CD46 [596]). Interestingly, MCP is a membrane-bound complement regulator that contains SCRs and belongs to the RCA family of proteins like C4BP, factor H, and FHL-1. The binding of M proteins to the MCP molecule is speculated to occur through the C-repeat domains and can be inhibited by factor H (596). In contrast, the binding of HEp-2 cells appears to require the N-terminal domain of the M6 protein (832). It is clear that other receptors are responsible for mediating streptococcal adherence to many other cell types including fibroblasts (597). Other proteins implicated in the adhesion of GAS to host tissues are the fibronectin binding proteins FBP54 (132) and SfbI/protein F (see below).

Antigenic variation of M proteins: evidence of horizontal gene transfer. A fundamental question in the biology of M proteins is how they evolve to vary their antigenic epitopes without disturbing the critical contacts necessary to maintain their structural and ligand binding characteristics. This question is particularly valid in light of the findings that the N-terminal hypervariable domains bind complement regulatory proteins. Both sequencing of several M protein genes and PCR analysis have allowed several laboratories to study the mechanisms by which sequence variation can occur. M proteins generate sequence diversity by point mutations, small insertions and deletions, and intragenic recombination between tandem repeat domains (212, 306, 347).

Analysis of several Mga regulons by PCR suggested that the evolution of the Mga regulons involved gene duplication followed by sequence divergence (344). Other studies have shown that the GAS also possess the ability to horizontally transfer gene segments, not only with group A organisms but possibly also with other streptococcal groups (420, 730, 849). Unusual mosaic genes that possess sequence elements from both *enn* and *emm* genes have been observed in three GAS strains, indicating that M proteins probably have the ability to recombine with one another (635, 849). Recently an insertion sequence has been identified within the Mga regulon of strain AP1, suggesting at least one possible mechanism by which the transfer of gene segments could occur (41). Another laboratory found another insertion sequence, *IS1548*, immediately downstream of the *scpA* gene in several GAS and GBS strains (277). It is possible that the considerable genetic diversity observed in the streptococcal Mga regulons is assisted by the presence of nearby insertion sequences.

The discovery of horizontal transfer of M-protein genes has challenged the previous assumption that the relatedness of GAS strains could be determined through the serologic and genetic properties of their M proteins. A comparison of 79 different M serotypes by multilocus enzyme electrophoresis revealed that the variation of the *emm* genes and overall genetic relationship between the various GAS strains are not congruent (848). This finding was supported by another study of several clinical M type 1 isolates that found that identity of *scpA* and *emm* genes between strains did not correlate with similarity in overall genetic structure (568). Therefore, the fact that two GAS strains possess identical or similar M serotypes does not by necessity indicate that the two strains are closely related. These findings have significant implications for the epidemiological study of GAS.

In summary, M proteins are multifunctional binding proteins that have evolved the ability to bind several of the common building block domains of eukaryotic plasma and matrix proteins including albumins, fibrinogens, fibronectins, Ig, plasmins, and proteins containing SCRs. These binding functions may serve the multiple purposes of protecting the bacteria from the various phagocytic mechanisms that exist within the host, assisting in the adhesion to certain cell types, generating an inflammatory response, and triggering the invasion of deeper tissues. The conserved C repeats may mediate adhesion to keratinocytes by binding MCP and/or factor H, but the binding properties of this domain are still the subject of considerable debate. Indeed, despite recent advances, the role of M proteins in protection from phagocytosis remains something of an enigma.

Fibronectin binding protein (protein F/Sfb I). Many different pathogenic bacterial species have the ability to bind fibronectin, and the ability of GAS to bind fibronectin was established long before genes encoding the protein receptors responsible for the binding had been cloned. Fibronectin itself is a very large, multifunctional glycoprotein that is found as a disulfide-linked, soluble dimer in most body fluids or in a matrix form that is insoluble and is a major component of both basement membranes and ECM. Fibronectin has multiple binding domains that are capable of binding to a wide variety of compounds such as collagens, fibrin, heparin, and actin. It stands to reason, therefore, that GAS would use this molecule as a target for binding because it is ubiquitous throughout the human body and is especially prevalent at sites, such as the dermis, where an infection would be initiated.

Many factors have been implicated in the binding of fibronectin to the surface of GAS. Although some early studies had suggested that lipoteichoic acid is responsible for the binding of GAS to fibronectin, later studies refuted those claims. There are reports in the literature of at least five streptococcal fibronectin binding factors including Emm3 (666) and protein H (230), SDH (614), OF/SfbII (448, 658), FBP54 (133), and protein F/SfbI (303, 778). Given the rather promiscuous binding abilities of fibronectin itself, the results of any in vitro binding study must be viewed with caution until the biological relevance of such an interaction can be clearly demonstrated.

The best-characterized group of streptococcal fibronectin binding proteins are the SfbI/protein F family members. SfbI and protein F are essentially identical fibronectin binding proteins that were cloned from different strains of GAS. Southern blot analysis of several streptococcal isolates has revealed that SfbI-like proteins are present in approximately 70% of GAS strains. The gene encoding protein F, *prtF*, was cloned by screening a streptococcal expression library in *E. coli* for the expression of a protein which could inhibit the binding of radiolabeled fibronectin to the surface of GAS (303). A role for this protein in binding fibronectin has been demonstrated, since mutants generated by insertional inactivation are unable to adhere to either fibronectin or respiratory epithelial cells (303) and the exogenous addition of purified protein F/SfbI is able to inhibit the binding of fibronectin to the surface of GAS. More convincingly, expression of protein F in both nonadherent streptococci and enterococci enables these bacteria to bind fibronectin and respiratory epithelial cells (304).

The SfbI/F proteins contain a rather large N-terminal leader peptide followed by a slightly variable aromatic region of approximately 200 amino acids and a set of conserved proline-rich repeat domains (RD1). The fibronectin binding region begins immediately downstream from RD1 and consists of a stretch of 43 amino acids (UR or UFBD) followed by up to five repeat domains (RD2) which are homologous in primary sequence to other fibronectin binding proteins including that of

S. aureus (720, 777, 778). Both the number of repeats and the sequence variation within the N-terminal variable region account for the heterogeneity between strains. Binding studies have demonstrated that the minimal functional unit of the RD2 domains is generated at the junction between two adjacent repeats and that the RD2 and UR domains of protein F bind different domains of the fibronectin molecule (609). A more recent study has indicated that protein F can also bind fibrinogen through the N-terminal variable domain that precedes the RD1 repeat domains (414).

C5a peptidase. One of the terminal steps of both the alternative and classical complement cascades is the proteolytic conversion of the full-length C5 protein into the biologically active C5a and C5b molecules by the C5 convertase complex. C5b plays a role in the membrane attack complex, which is generally ineffective against gram-positive bacteria. C5a, on the other hand, is a potent chemoattractant that recruits PMNs to the site of infection (360). To inhibit the recruitment of these PMNs, the streptococci produce a protease that proteolytically inactivates C5a (845, 846).

The gene for the C5a peptidase (scpA) encodes an 1,167-residue protein with a molecular mass of approximately 128 kDa (118). A 31-residue N-terminal leader peptide and a very large N-terminal catalytic domain are followed by four short repeats that lie almost immediately upstream of the cell wall sorting signal. The enzymatic domain bears significant homology to the subtilisin family of proteases, but, unlike the broad activities displayed by many members of this class of enzymes, the C5a peptidase shows remarkable specificity by being unable to cleave the full-length C5 molecule and cleaving only a single bond within C5a (125).

Insertional inactivation of the *scpA* gene demonstrated that the C5a peptidase plays a role in virulence in a mouse model (386). Mutant strains were cleared more efficiently than wild-type strains were. In addition, the mutant strains were trafficked to the lymph nodes whereas the wild-type strain was found predominantly in the spleen. The C5a peptidase has been investigated as a vaccine candidate due to its widespread conservation throughout GAS (385). Genes nearly identical to *scpA* have also been found in both group B and G streptococci, although the expression of *scpA* in the group G streptococci appears to be restricted to the specific strains that are capable of infecting humans (119, 123, 124).

Serum opacity factor/SfbII. OF is an apoproteinase responsible for the ability of some strains of GAS to cause opalescence in serum (703). As mentioned above, the expression of OF is usually restricted to GAS strains that express class II M proteins. The cloning of OF in 1995 by two independent groups showed unequivocally that OF is distinct from M proteins (448, 658).

Whereas Rakonjac et al. cloned the OF gene on the basis of its ability to cause opalescence in serum (658), Kreikmeyer et al. cloned the gene based on its ability to bind fibronectin (448) and hence named the protein SfbII. The OF/SfbII protein itself is very large (1,025 residues) and contains an N-terminal leader peptide and a C-terminal cell wall sorting signal with a seryl residue substituted instead of the threonyl of the LPXTG motif. A domain repeated 2.5 times near the C terminus of the protein is homologous to the RD2 repeats from SfbI/protein F, and purified fusion proteins containing these domains are capable of competitively inhibiting the binding of radiolabeled fibronectin to streptococcal cells. Both groups noted that the gene is present only in a subset of streptococcal isolates, which correlates with what was previously known about OF (see above).

T antigen. T antigens form a group of approximately 25 serologically distinct surface molecules found on the surface of group A, B, C, and G streptococci that have the common property of being resistant to digestion with trypsin (437, 469). Antibodies against T antigens are not protective, and therefore very few studies on the biological relevance of these molecules have been carried out. Although T antigens are expressed independently of M antigens and more than one T antigen can be expressed simultaneously on the same strain, most M types are associated with a single T type (389). T antigens have been used as a means of subclassifying different strains of GAS and are used in cases where M antigen, due to either a lack of available typing sera or a lack of expression, cannot be used. Although antibodies to T antigen are generated during the course of an infection, these antibodies are not protective against subsequent infections.

Although two T antigens have been purified (390, 502), to date the nucleotide sequence of only one T-antigen gene, *tee6* from the M6 serotype strain D471, has been determined (714). The *tee6* gene encodes a 55-kDa polypeptide of 537 amino acids (T6) that possesses a C-terminal cell wall sorting signal. The protein is rich in serine, threonine, and lysine residues and is rare among the streptococcal surface proteins in containing no discernible repeat domains. DNA hybridization studies demonstrated that the 5′ end of the *tee6* gene hybridized with chromosomal DNA of only 10 of the 25 known T types, and probes constructed from the 3′ end of *tee6* hybridized with even fewer isolates (397). Indeed, chromosomal DNA from several T-type isolates failed to hybridize with any *tee6* probe. These findings leave open the possibility that the T antigens are actually several different molecules that have in common only their ability to resist trypsin digestion.

Streptococcus agalactiae (Group B Streptococci)

Group B streptococci (GBS or *S. agalactiae*) are responsible for the majority of cases of neonatal sepsis and meningitis in developed countries (25). GBS are frequently present in the vaginal flora of humans and can be transmitted to the neonate during or before birth. GBS can also cause invasive infections in adults, particularly the immunocompromised and the elderly (195). GBS can be subclassified immunologically according to their polysaccharide capsules, which fall into at least nine distinct types (436, 842). A majority of the invasive GBS infections are due to strains expressing the type III capsular polysaccharide, although strains expressing other capsular types are increasingly gaining in importance (66). The GBS can also be grouped according to the expression of a set of surface proteins designated Cα (alpha), Cβ, and Rib (202). In experiments with animal models, it has been determined that antibodies against either the polysaccharide capsule or the surface proteins are protective against infection with GBS (52, 685, 751, 843). Capsular polysaccharides can be poorly immunogenic, however, and this has led many laboratories to focus on the use of surface proteins as possible vaccines (471, 842). Unfortunately, research on the GBS surface proteins has focused primarily on their vaccine potential and relatively little has been determined about the role of these proteins in virulence.

Cα (alpha, alpha C) and Rib. Cα and Rib are homologous trypsin-resistant proteins found on the surface of the GBS. Rib is almost always found on the more invasive GBS strains of capsular type III, whereas Cα is usually expressed on strains expressing other capsular types (220, 751). Recently another Cα-like protein has been identified on a type V strain, but the sequence of this protein has not yet been published (464, 465).

Rarely, if ever, are Cα and Rib expressed together on the same cell (220). The genes encoding Cα (*bca*) and Rib (*rib*) were sequenced from the type Ia GBS strain A909 and the type III strain BM110, respectively (550, 837). The two proteins have a similar organization with unusually long leader peptides followed by N-terminal domains of ≈170 amino acids that are 61% identical to each other. The N-terminal domains are followed by set of 9 (Cα) or 12 (Rib) tandem repeat domains that in both cases are followed by a classical C-terminal cell wall sorting signal. The repeat domains of Cα and Rib contain 82 and 79 amino acids, respectively, and are 47% identical to one another. Strikingly, there is no variation between individual repeats in either protein, even at the nucleotide level. In both cases the repeat domains constitute greater than 70% of the total length of the protein.

Both Cα and Rib proteins are capable of eliciting protective immunity against GBS, but immunity against one does not confer immunity against GBS strains expressing the other even though the two proteins are highly homologous (471, 751). Since almost all strains of GBS express either alpha or Rib, a vaccine composed of both proteins should, in theory, provide broad-range immunity against these bacteria. The number of repeat domains among Cα proteins of different clinical isolates of GBS can vary significantly but averages between 8 and 9 (509). It is apparent that the repeat domains in both Cα and Rib are subject to insertions and deletions due to homologous recombination. There is some evidence that strains of GBS expressing Cα antigens with fewer repeat domains are less susceptible to the host immune response than are those that express Cα antigens containing larger numbers of repeats (280, 510). Conversely, it has been shown that Cα proteins with fewer repeat domains are more immunogenic (278, 279). It would therefore appear that the selective advantage conferred by Cα molecules containing fewer repeats is counterbalanced by their ability to generate a more vigorous immune response.

The role of Rib and Cα in the pathogenesis of GBS is not understood; however, a recent experiment has shown that a mutant GBS strain in which the *bca* gene was insertionally inactivated is attenuated in its ability to cause disease in mice (483). More studies are necessary to determine the exact role of these proteins in virulence.

Cβ (beta antigen, Bac, IgA binding protein). The Cβ antigen is found on less than half of GBS strains, and many of these strains appear to express a truncated, secreted form of the protein (81, 512). The role of Cβ in virulence is unclear, but the protein has a high affinity for serum IgA and, under some conditions, secretory IgA (52, 81, 487, 691). Immunization of pregnant mice with purified Cβ can confer resistance against infection with GBS onto neonatal pups (511). The gene encoding Cβ (*bac*) was cloned from GBS strains SB35 (319) and A909 (382) by two independent laboratories. Both genes possess a 37-residue leader peptide and a classical C-terminal cell wall sorting signal. Approximately 180 residues upstream of the sorting signal is an unusual domain composed almost entirely of a triplet repeat, in which the first residue is a proline, the second residue alternates between a positively and a negatively charged amino acid, and the third residue is uncharged. The two genes differ slightly in the proline-rich repeat region, with that from the SB35 strain containing two short deletions. Analysis of recombinant Cβ expressed in *E. coli* indicated that the Cβ protein contains two or more separate IgA binding domains within the N-terminal half of the molecule, designated A and B (382). A later study mapped the binding site of the A domain to a region centered around a hexapeptide motif (MLKKIE), but it was not conclusively determined that this motif actually participated in the binding of IgA (383). Neither IgA binding domain on Cβ bears any significant homology to the IgA binding domains found in M proteins (50, 391). The Cβ typing antisera appear to recognize a separate domain that lies C-terminal to the IgA binding domain(s) (319).

Group C and G Streptococci

Streptococci in Lancefield groups C and G (GCS and GGS) comprise a group of closely related species that are capable of causing disease in both humans and a wide variety of animals. The large-colony forms of GCS, including the alpha-hemolytic *S. dysgalactiae* and beta-hemolytic *S. equi* (subspp. *equi*, *equisimilis*, and *zooepidemicus*), are common veterinary pathogens. Less often, these strains will infect humans, with *S. equi* subsp. *equisimilis* (*S. equisimilis*) being the most frequently isolated along with the small-colony form, *S. anginosus* (56). GGS are generally not given a species designation and are also associated with disease in both humans and animals.

M protein. Clinical isolates of GCS and GGS can possess antiphagocytic M proteins similar to those found in GAS (56, 57, 129, 395, 745). However, several studies have suggested that the expression of such proteins is limited to the specific strains that can infect humans (555, 717, 731). As mentioned previously, the GGS and GAS appear to be able to share their M protein genes by horizontal transfer (730, 750). A recent study of 38 human GGS isolates revealed that each possessed a single class I *emm* gene that could be divided into one of 14 distinct types based on the N-terminal sequence of its protein (717). The sequences of some of these proteins was similar to known N-terminal sequences from GAS M proteins, whereas others were found to be quite divergent.

SzP and SeM. *S. equi* and *S. zooepidemicus* frequently colonize the nasopharynx and genital mucosa of horses. *S. equi* is the causative agent of strangles, a highly contagious disease of horses, whereas *S. zooepidemicus* is a normal mucosal commensal organism that can cause disease in an opportunistic fashion. Recent genetic studies have indicated that *S. equi* (also known as *S. equi* subsp. *equi*) is actually a variant strain of the more genetically diverse *S. zooepidemicus*. Strains of *S. zooepidemicus* express an acid-extractable surface antigen (SzP) that, like the GAS M proteins, is antigenically variable among strains. In contrast, *S. equi* appears to express two invariant surface proteins, one of which is related to SzP while the other appears to be unique to this strain (SeM).

The genes encoding the SzP protein have been sequenced from *S. equi* (SzPSe) and *S. zooepidemicus* W60 (SzPW60), as has the gene encoding SeM (784, 785). Both SzP and SeM bind equine fibrinogen, and it appears that these proteins can limit the deposition of equine C3 on the bacterial surface (72, 73, 784). Antibodies against SzP are opsonigenic against *S. zooepidemicus* (but not against *S. equi*), whereas *S. equi* is opsonized by antibodies against SeM (784). For these and other reasons, the SzP and SeM proteins were designated "M-like." However, despite their functional similarities and the presence of an N-terminal leader peptide and C-terminal cell wall sorting signal, SeM and SzP do not bear significant homology to each other or to the M proteins of GAS. SzPSe and SzPW60 are 85% homologous to one another and do not possess large tandem repeats; however, the proline-rich region of each is composed of several copies of a 4-residue repeat, PEPK. SeM contains two tandem repeats of 21 residues each and several shorter C-terminal tandem repeats. No specific functions have yet been ascribed to any domain of either SeM or SzP.

Protein G and related IgG binding proteins (protein G, MIG, MAG, ZAG). As mentioned above, Myhre and Kronvall, in 1977, classified the IgG binding activities of the gram-positive bacteria, giving the distinction "type III" to proteins with binding properties similar to those found on the GCS and GGS (569). Shortly thereafter, Myhre and Kronvall also identified an albumin-binding activity on the surface of the GCS and GGS (569a). It has since become apparent that a single protein, protein G, confers both the IgG and albumin binding properties on these bacteria. Although possessing multiple ligand binding properties similar to those found in some GAS M proteins, protein G and related proteins are structurally distinct from the M proteins. Over the past decade, protein G-like molecules have been identified in several group C and G species of human and bovine origin as well as in *S. dysgalactiae* (MIG and MAG) (398, 399, 401), *S. zooepidemicus* (ZAG) (400), and the distantly related anaerobe *Peptostreptococcus magnus* (see below).

Protein G was first isolated based on its IgG binding activity from the group G strain G148 (60) and from the group C strain C26RP66 (667, 668). The albumin binding properties of this molecule were not described until the gene had been isolated and expressed in *E. coli* (59). Genes encoding protein G have been sequenced from strains G148 (290, 603), GX7805 (204), GX7809 (194), G43, and the group C strain C43 (734), allowing a detailed comparison of their structures. Unlike the GAS M proteins, the binding activities of the protein G family are contained within distinct modular domains, whose number can vary from strain to strain. The globular structures of these domains are more reminiscent of staphylococcal protein A (see below) than the extended alpha-helical coiled-coil structure of the M proteins.

The binding of albumin and that of IgG occur within separate domains of protein G, each encoded by a set of tandem repeats (6, 735). The modular albumin binding domains (GA modules) are contained within the N-terminal region of protein G, whereas the IgG binding domains are encoded in a more C-terminal region of the molecule. The structures of both domains have been determined (286, 387, 388). GA modules are found in a wide variety of streptococcal and peptostreptococcal albumin binding proteins; however, these domains are not present in all protein G molecules from human clinical isolates (388, 733, 734). Protein G also binds the protease inhibitor α_2-macroglobulin (α_2M), but this binding is inhibited by IgG and it is unclear whether it is physiologically relevant (736).

MIG and MAG are surface proteins cloned from two different strains of *S. dysgalactiae*, whereas ZAG was isolated from a strain of *S. zooepidemicus* (399–401). Earlier studies had suggested that *S. zooepidemicus* possessed a unique type (type V) of IgG binding protein; however, sequence analysis revealed that MIG, MAG, and ZAG are similar to type III protein G. Like protein G, MAG and ZAG bind albumin (MIG does not), and all three of these proteins have been shown to bind α_2M. The binding of α_2M occurs through an N-terminal domain unique to MIG, MAG, and ZAG that is not found in protein G.

Fibronectin binding proteins. Fibronectin binding proteins have been cloned from the GCS strains *S. dysgalactiae*, *S. equisimilis*, and *S. zooepidemicus* (490–492). *S. dysgalactiae* has two adjacent genes, *fnbA* and *fnbB*, that encode fibronectin binding proteins (490). Unlike the two fibronectin binding proteins from *S. aureus*, however, FnbA and FnbB are quite dissimilar and are most probably not the product of gene duplication. In fact, the C-terminal fibronectin binding domains of FnbA and FnbB each have greater homology to protein F than they do to one another. The reasons why this bacteria contains two proteins with this activity are unclear, although under laboratory conditions only FnbB appears to be expressed (490). The C-terminal domain of FnbB is also highly homologous to the fibronectin binding protein of *S. equisimilis* (491).

The fibronectin binding protein of *S. zooepidemicus*, FNZ, is structurally similar to protein F, including the presence of nearly identical fibronectin repeat domains and the presence of an UR-like sequence. The UR domain of FNZ, however, lies between the first and second fibronectin binding repeats rather than immediately upstream as is found in protein F. FNZ, at ≈60 kDa, is rather small in comparison to the rather large (>100-kDa) fibronectin binding proteins of *S. dysgalactiae* and *S. equisimilis* (492).

Streptococcus pneumoniae

The pneumococci are one of the leading causes of septicemia, meningitis, and lower respiratory tract infections in humans. Despite rapid and aggressive antibiotic therapy, pneumococcal infections can lead to a variety of debilitating complications including hearing loss. The polysaccharide capsule expressed by these bacteria is required for virulence but in itself is not toxic. Although proteins are obviously important, their role in the pathogenicity of these bacteria has not been extensively studied (620). *S. pneumoniae* produces two hemolysins, one of which, the well-studied pneumolysin, has been clearly demonstrated to contribute to the disease process (38, 109, 620, 663). In addition, the pneumococci produce two adhesins, PspA and PsaA, which may also contribute to the ability of these bacteria to establish an infection within a host (48, 875). Only two cell wall-anchored enzymes, a neuraminidase and a β-*N*-acetylglycosaminidase, have thus far been cloned in *S. pneumoniae*. Both of these glycosidases probably play a role in virulence as well.

Neuraminidase. It has long been appreciated that all clinical isolates of *S. pneumoniae* possess neuraminidase activity (425, 608). Neuraminidases, found in a wide variety of pathogenic microbes, cleave terminal sialic acid residues from a variety of glycosylated proteins, lipids, and oligosaccharides found both in body fluids and on the surfaces of cells. Neuraminidase activity correlates with virulence in pneumococcal meningitis, and early studies with crudely purified pneumococcal neuraminidase demonstrated its toxicity in mice (426, 608). At least two genes encode neuraminidases in the pneumococci (47, 108). One gene, *nanB*, encodes a putative secreted protein, while a gene 4.5 kb upstream, *nanA*, encodes a protein with the typical characteristics of a gram-positive surface proteins (47, 107). NanA and NanB have no significant homology to one another and appear to have optimal activities at different pH values, suggesting that they may act at different sites or stages during an infection (47). NanA is >100 kDa and has four copies of a consensus bacterial neuraminidase sequence motif (SXDXGXTW), suggesting that it belongs to the "large" family of bacterial neuraminidases (107, 675). The surface localization of NanA has been confirmed by immunoelectron microscopy (107).

The exact role(s) that the neuraminidases play in virulence is unclear, although it has been suggested that removal of sialic acid from surface molecules can "unmask" carbohydrate ligands for pneumomcoccal adhesion (449, 489). A recent experiment demonstrated that a strain of *S. pneumoniae* with an interrupted *nanA* gene was not defective in its ability to cause cochlear damage in an experimental guinea pig meningitis model whereas strains deficient in pneumolysin were avirulent (856). These strains, however, still possessed an intact *nanB* gene, although the authors claimed that the activity from this gene was minimal.

β-*N*-Acetylhexoseaminidase. The gene for the pneumococcal β-*N*-acetylglucosaminidase (*strH*) was cloned from a phage lambda expression library from *S. pneumoniae* by screening for plaques with enzymatic activity (122). StrH is very large (144 kDa) and possesses both an N-terminal leader peptide and a typical C-terminal cell wall sorting signal. The protein also contains two rather large (335-residue) tandem repeat domains, which account for approximately half of the mature protein. The repeats, separated by approximately 100 residues, each contain a 30-residue sequence motif that is homologous to conserved sequences found in other bacterial hexosaminidases. Host tissues are abundant in surface molecules containing GlcNAcβ1-4-linked residues, a substrate for the enzyme, but the role of the StrH protein in the biology of *S. pneumoniae* is unclear.

Oral Streptococci (Viridans Group)

The oral cavity is typically host to several species of gram-positive bacteria, most notably the viridans group streptococci. Important among the viridans group are *S. mutans* and *S. sobrinus*, which have been implicated as primary causes of dental caries (376, 497). Colonization of the oral cavity requires that the bacteria bind to salivary components, to other bacteria, and to both soluble (dextran) and insoluble (mutan) extracellular polysaccharides that are synthesized by the bacteria themselves. Surface hydrophobicity has been speculated to play a role in the ability of bacteria to adhere to the oral pellicle, and surface proteins have been implicated in contributing to overall surface hydrophobicity.

Surface proteins from these organisms have been widely studied for their role in oral colonization as well as their potential as vaccine targets. At least 20 polypeptides are exposed on the cell surface of *S. gordonii* and *S. sanguis* (14, 377). Sequence analysis of several of these surface proteins has revealed that viridans streptococci use similar adhesins to attach to both host tissue components and other bacteria (160).

Antigen I/II (P1 protein, B antigen, PAc, IF, sr, SpaA). The streptococcal antigen I/II proteins have been found on the surface of almost every oral streptococcal species studied thus far. Antigen I/II (688) of *S. mutans* serotype c has also previously been designated B antigen (689), IF (359), P1 (221), MSL-1 (150), and PAc (598). Genes homologous to antigen I/II have been cloned and sequenced in *S. mutans* serotype f (sr protein; [1]), *S. gordonii* (SspA and SspB [149]), and *S. sobrinus* (SpaA [94] and pag).

These proteins are almost identical and have both N-terminal leader peptides and C-terminal cell wall sorting signals. They are large (1,500 to 1,566 amino acid residues) and, like many other surface proteins, contain multiple binding activities and repeat domains (378). I/II antigens have been reported to be able to bind salivary glycoproteins as well as other oral microbes. The structure and function of this group of proteins have been the subject of several recent reviews (375, 376, 378).

The surface localization and cell wall attachment of P1 to the streptococcal cell wall has recently been demonstrated (353, 565, 566). A mutation was isolated in *S. mutans* GS-5 that resulted in the complete loss of surface attachment of P1 (565). Sequencing of the GS-5 variant revealed a frameshift mutation that resulted in the production of a I/II variant which was lacking the C-terminal end. Homonylo-McGavin and Lee found that antigen I/II of *S. mutans* could be localized to the surface of *S. mutans*, *S. gordonii*, and *Enterococcus faecalis* only if the C-terminal domain was intact (353). In addition, antigen I/II remained associated with the cell after boiling in SDS only if the cell wall sorting signal was present. This was confirmed in a later study in which it was shown that removal of the charged tail led to the complete solubilization of antigen I/II from the cell surface (566).

CshA of *S. gordonii*. CshA is a very large (≈290-kDa, 2,508-amino-acid) surface protein which was identified in *S. gordonii* (538, 539). Cells defective in the expression of this protein have decreased cell surface hydrophobicity and a defect in their ability to coaggregate with oral actinomycetes (538, 539) and other bacteria (537), suggesting that this protein is probably necessary for colonization and adherence to the oral pellicle. Indeed, *cshA* mutants are defective in their ability to colonize the oral cavity of mice (539).

Like many other surface proteins, much of CshA is composed of repeating domains (539). The N-terminal domain of the mature protein (amino acids 42 to 878) is nonrepetitive, whereas the central portion of the protein (amino acids 879 to 2417) is composed of 16 repeat regions of ≈100 amino acids each. All of the binding regions thus far identified in CshA have been mapped by antibody inhibition studies to the N-terminal nonrepetitive domain (537). The C terminus of CshA is necessary for the retention of the polypeptide on the cell surface, since C-terminal mutations which truncate CshA to a protein of ≈260 kDa result in the complete secretion of CshA into the growth medium and a subsequent loss of surface hydrophobicity (538).

β-D-Fructosidase (FruA) of *S. mutans*. Oral streptococci produce polysaccharides that have a short half-life in dental plaque and are thought to be used for short-term sugar storage. These polymers are synthesized by a set of glucosyl-transferases (see below). Unlike the insoluble mutan polysaccharides, which are composed of α1-3 linkages and are unable to be enzymatically degraded, the storage polysaccharides are composed of glycosidic bonds that are susceptible to enzymatic lysis. β-D-Fructosidase (FruA) is a surface-associated enzyme capable of liberating fructose through the degradation of levans as well as sucrose, raffinose, and inulins. The *fruA* gene was cloned and sequenced from *S. mutans*, but Northern blot analysis suggests that homologues are likely to exist in all of the mutans streptococci (103). FruA is notable in having almost no proline or glycine residues in the area of the protein predicted to span the cell wall.

Interestingly, a study of *S. mutans* grown in a chemostat demonstrated that over 95% of the fructosidase activity was located in the supernatant (105) whereas a vast majority of the activity was cell associated in batch culture (103). An investigation into this phenomenon revealed that most of the fructosidase can be found in the supernatant when the bacterium is grown at a pH of 7.0, whereas approximately half is cell surface bound when the organism is grown at a pH of 5.0. The study also demonstrated that the release of fructosidase into the extracellular media can be inhibited by copper, a phenomenon also observed for the release of antigen I/II (104).

Dextranases of *S. mutans* and *S. sobrinus*. Cell surface dextranases cleave glycosidic bonds and are thought to be involved both in the metabolic utilization of sugar and in controlling the amount and content of extracellular polymerized glucans (126, 249). The streptococcal dextranases degrade dextran into isomaltosaccharides (362). Genes encoding extracellular dextranases have been cloned from *S. mutans* (363), *S. sobrinus* (827), *S. salivarius* (594), and *S. suis* (723), although only the dextranases from *S. mutans* and *S. sobrinus* possess C-terminal cell wall sorting signals. The *S. mutans* and *S. sobrinus* dextranases are 47% homologous to one another, although the *S. sobrinus* enzyme is somewhat larger. In addition, the two enzymes have similar enzymatic properties and pH optima, indicating functional similarity (827). Inactivation of the dextranase gene of *S. mutans* appears to increase the ability of the bacteria to adhere to smooth surfaces but has no effect on sucrose-dependent cell-cell aggregation (126).

Glucan binding protein of *S. mutans*. Under conditions of stress, certain strains of *S. mutans* express a glucan binding protein that enables these cells to aggregate in the presence of dextran (704). The gene for this protein, *gbpC*, has recently been cloned and has been shown to encode a ≈60-kDa protein that contains both an N-terminal leader peptide and classic C-terminal cell wall sorting signal (704). The glucan binding activity of GbpC has been demonstrated in extracts of *E. coli* which harbor a plasmid encoding the gene (704). GbpC has limited homology to streptococcal antigen I/II, and the protein cross-reacts with anti-antigen I/II antisera. More work is required to determine why there appear to be several glucan binding proteins in *S. mutans*. The only other glucan binding protein to be sequenced thus far has no C-terminal cell wall sorting signal (26).

WapA (protein antigen III) of *S. mutans*. Wall-associated protein antigen A (WapA [201, 689]) and antigen III (686) were recently shown to be identical (687). WapA is a 45-kDa surface antigen of *S. mutans*, which has been implicated in helping the bacteria to bind smooth surfaces and to undergo sucrose-dependent aggregation (655), although this is a matter of some dispute (307, 687). Also disputed is its ability to serve as an effective vaccine against dental caries (687).

Fimbriae of *Actinomyces*

The actinomycetes, including *Actinomyces naeslundii* and, perhaps, *A. odontolyticus*, are some of the most common bacteria in the oral flora. Like the oral streptococci, these bacteria are involved in the early stages of plaque development and have the ability to bind both the tooth surface and other oral bacteria (441, 484). *A. naeslundii* binds to the tooth surface via a fimbrial structure, designated type 1, which has affinity for proline-rich proteins that coat the tooth enamel (121, 257). The adherence of actinomycetes to eukaryotic host cells and other bacteria, including several streptococcal species, is directed by a separate set of fimbriae designated type 2. Type 2 fimbriae appear to utilize a lectin-like activity to bind to the surface of target cells, and this binding can often be disrupted by high concentrations of sugars such as lactose (298, 440).

Strains of *A. naeslundii* may express either one or both types of fimbriae on their surface. One strain, T14V, expresses both types of fimbriae and has been used as a model system for the study of these structures in detail at the molecular level (120). The gene encoding the type 1 fimbrial subunit (*fimP*) of strain T14V has been cloned and sequenced, as has the gene encoding the type 2 fimbrial subunits (*fimA*) of T14V and another strain, WVU45 (867, 868). All three cloned subunit genes encode proteins of ≈54 to 59 kDa that have typical N-terminal leader peptides as well as C-terminal cell wall sorting signals. The type 2 subunit from T14V has a very high degree of homology (≈65% identity) to the type 2 subunit from WVU45 but only moderate homology (≈31% identity) to the type 1 subunit from T14V (868, 869).

The presence of the cell wall sorting signals is somewhat unexpected, and their possible role in fimbrial assembly is unclear. Accessory genes that appear to be involved in the assembly of intact fimbriae from individual subunits have been identified for both the type 1 and 2 fimbriae from strain T14V, but no pathway for assembly has been determined and specific roles for these genes have not yet been determined (869, 870). A clue to the role of the C-terminal cell wall sorting signal may be revealed by the fact that it is not possible to dissociate assembled fimbriae into their individual monomer subunits, suggesting that they may be covalently linked to one another. Additionally, antibodies raised to the C-terminal domain of a recombinant monomer subunit are unable to recognize the intact fimbriae, suggesting that the C-terminal domain may be proteolytically removed (869). This evidence, although far from conclusive, has led Yeung et al. to suggest that perhaps the C-terminal sorting signal directs the covalent multimerization of the fimbrial subunits (869). If this were true, it would represent a novel use of the sorting machinery to synthesize a complex fimbrial organelle. Obviously the verification of this model will require the structural characterization of both free and complex-bound fimbrial subunits.

Protein L and PAB of *Peptostreptococcus magnus*

The gram-positive anaerobe *Peptostreptococcus magnus* is a common human commensal organism that can also cause a variety of diseases including vaginosis and urethritis. Approximately 10% of clinical isolates express a unique Ig receptor, protein L (PPL), that has the unusual ability to bind the variable portions of Ig kappa light chains (4, 58, 188, 587, 855). This unusual binding specificity confers upon PPL the ability to bind antibodies of almost any subclass, and therefore this molecule has generated much interest as a tool for purifying and studying Ig. The gene encoding PPL has been cloned and sequenced from *P. magnus* 312 (413) and 3316 (567). A comparison of their structures reveals that these proteins have a modular design similar to that found for protein G from the GGS. PPL_{3316} and PPL_{312} possess four and five tandem repeat domains, respectively, that encode the Ig light-chain binding modules, whose three-dimensional structure has recently been determined (854). The two proteins also possess common tandem repeat domains of unknown function. PPL_{3316}, but not PPL_{312}, possesses four copies of the albumin binding GA modules similar to those found in protein G, and, as expected, strain 3316 binds albumin whereas strain 312 does not (413, 567).

A physiological role for the light-chain binding function of PPL has been proposed. Addition of strain 312 to a culture of human basophils was found to stimulate the release of histamines, whereas no such stimulation was observed upon the addition of a strain that lacks PPL (619). A similar effect was observed upon the addition of purified recombinant PPL to the cultures, presumably due to cross-linking of the membrane-bound IgE receptors present on these types of host cells.

Approximately half of *P. magnus* strains that do not possess PPL bind albumin through a receptor known as PAB. The gene encoding PAB has been sequenced from strain ALB8 and was found to encode a 43-kDa protein that, like protein G and PPL, is strikingly modular in its design (141). Although PAB lacks the kappa light-chain binding domains found in PPL, it does have many similarities to PPL, including the presence of a "GA module" as well as a sequence homologous to one of the repeats of unknown function from PPL_{312}. The structural similarities between PAB and PPL seem to indicate that these two proteins are actually derived from a common ancestral molecule that evolved through the shuffling of the relevant binding modules (140). Indeed, a recent study has identified short nucleotide sequences within PPL and PAB that appear to facilitate the shuffling of these modular domains in a manner similar to the swapping of exons in eukaryotic cells (140).

Enterococci

Enterococci possess a unique and highly efficient mechanism for the conjugal transfer of plasmid DNA (166, 178). Expression of this transfer system in donor cells is induced by an oligopeptide mating pheromone that is secreted by recipient cells (167). Induction results in the increased expression of surface protein (aggregation substance [AS]) on the donor that acts as an adhesin specific for the surface of recipient cells (239). An additional surface protein (surface exclusion protein) is involved in preventing donor cells from conjugating with identical donors (170). The pheromone-induced expression of aggregation protein appears to be controlled by a novel posttranscriptional mechanism (36, 37). The genetics of this system has recently been reviewed in detail (168, 169). A similar conjugative plasmid transfer system has recently been identified and sequenced in the *Enterococcus faecium* plasmid pHKK701 (315).

Aggregation substance. The genes encoding AS from three conjugal plasmids, pCF10 (411), pAD1 (237), and pPD1 (236), have been sequenced, and it is believed that almost all enterococcal conjugal plasmids encode similar aggregation proteins (238, 339, 857). The three known AS proteins are all large (≈145-kDa) proteins that have significant sequence homology to each other and possess a typical C-terminal cell wall sorting signal as well as a proline-rich domain. The domain structure of these proteins is similar to that found in both the antigen I/II proteins of the viridans streptococci and the CluA protein of *L. lactis*, consistent with their roles as mediators of bacterial aggregation. The ligand for AS, designated enterococcal binding substance (EBS), is unidentified to date. The inactivation of several unlinked genes is required to obtain an EBS-negative phenotype, and it is speculated that lipoteichoic acid may be a component (711). The expression of both AS and EBS has been suggested to play a role in virulence in addition to their role in conjugal transfer of DNA (602, 711).

Surface exclusion protein. As mentioned above, surface exclusion proteins reduce the probability of mating between enterococci carrying identical plasmids, although the exact mechanism underlying this phenotype is unclear. The surface exclusion proteins of the plasmids of pAD1 (840) and pCF10 (411) have been sequenced. Both genes encode homologous 95-kDa proteins that have both N-terminal leader peptides and C-terminal cell wall sorting signals. The gene encoding the surface exclusion protein of plasmid pPD1 has also been sequenced and has been found to contain an insertion sequence that results in a truncated protein without a C-terminal cell wall sorting signal (339). Cells containing plasmid pPD1 lack the surface exclusion phenotype, strongly suggesting that cell wall anchoring is required for the surface exclusion phenotype (339).

Lactococci

CluA. Not unlike their close relatives the enterococci, certain strains of lactococci are able to exchange genetic information through conjugal mating. Recently a gene encoding the aggregation factor was cloned from *Lactococcus lactis* subsp. *cremoris* (271). The gene encodes a polypeptide of 1,243 residues that has a modular structure similar to other surface proteins. Sequence analysis revealed homology to the aggregation proteins of *E. faecalis*. Three central domains have homology to both the *E. faecalis* domains and the antigen I/II proteins from the viridans streptococci.

NisP. Lantibiotics are ribosomally synthesized antimicrobial oligopeptides that are produced by several gram-positive species. These (β-methyl)lanthionine-containing peptides display activity primarily against other gram-positive bacteria and have been the subject of considerable interest because of their ability to inhibit food spoilage (147). Nisin, produced by *L. lactis*, is one of the best characterized of the lantibiotics. The genetics and modification of nisin and other lantibiotics have recently been the subject of several reviews (154, 190, 373, 693).

Nisin is synthesized as a 57-amino-acid precursor peptide in the cytoplasm, at which point it undergoes a series of posttranslational modifications before being exported from the cell. The N-terminal 23 amino acids of pre-nisin serve as an atypical leader peptide that assists in the secretion of pre-nisin from the cytoplasm by a putative ATP binding cassette transporter (186, 451). The exported nisin pre-peptide is inactive due to the presence of the N-terminal leader sequence but is converted to an active form upon removal of its leader peptide by a surface-located serine protease (product of the *nisP* gene). NisP has also been implicated in providing immunity to nisin, although the mechanism by which this would occur is unclear (866).

Sequence analysis of NisP has revealed that it contains both an N-terminal leader sequence and C-terminal cell wall sorting signal (810). Neither the surface localization nor attachment of NisP to the peptidoglycan has been verified experimentally. It is unclear why such an attachment would be necessary, given that functionally homologous lantibiotic-peptidases from other species do not have a cell wall sorting signal in their sequence (154).

PrtP. During growth in milk, the primary source of amino acids for the lactococci are the milk proteins (caseins), since the supply of free amino acids in milk is relatively limited (405). The caseins are initially broken down by the bacterium into small peptides, which are subsequently taken up by an oligopeptide permease system (453). The imported peptides can then be further degraded into individual amino acids by a series of intracellular proteases. The complete protease system of *L. lactis* has recently been reviewed (453).

The casein degradation pathway is initiated by a cell wall-bound serine protease called PrtP or CEP (192, 744). The *prtP* gene has been cloned and sequenced in a number of *L. lactis* strains (155, 429, 439), and each displays >95% homology to the other *prtP* genes. Sequence analysis has revealed that the proteins encoded by *prtP* are approximately 200 kDa and contain both an N-terminal leader peptide and a C-terminal cell wall sorting signal. The substrate specificity of the PrtP proteinase varies from strain to strain (192) but generally falls into one of two classes, designated PI and PIII, depending on the specific casein that the enzyme preferentially degrades (453). It has been demonstrated that PrtP is synthesized as a preproenzyme and, after being exported from the cytosol, is activated by the action of another cell surface protease, PrtM (293, 821), which is a lipoprotein (294). The attachment of PrtP to the cell wall and the role of the C terminus had been suggested several years before the cell wall sorting mechanism had been elucidated for protein A in *S. aureus* (155, 821).

Interestingly, a distantly related gram-positive bacterium, *Lactobacillus paracasei*, has a surface protein (PepP) which is homologous to the lactococcal PrtP. The putative C-terminal cell wall sorting sequence is unusual in that the ultimate glycine of the consensus motif has been replaced with an alanine (LPKTA) (343). It remains to be proven whether this sorting signal is functional.

Listeria monocytogenes (InlA, InlC2, InlD to InlF)

Listeria monocytogenes is an invasive intracellular pathogen that can infect animal hosts from within the intestinal lumen. Once inside the host, these bacteria invade eukaryotic cells and replicate intracellularly, thereby escaping clearance by the humoral immune response (131). The ability of *L. monocytogenes* to enter eukaryotic cells has been traced to a family of surface and secreted proteins, the internalins. Thus far, seven members of the internalin family have been found to exist (InlA to InlC, InlC2, and InlD to InlF) and all have been found to share certain structural features (162, 235). All feature a N-terminal leucine-rich-repeat domain (LRR) followed by a conserved inter-repeat region (IR) (Fig. 15). InlA, InlC2, and InlD to InlF possess C-terminal cell wall sorting sequences at their C termini; InlB is targeted to the cell envelope (see below) (82); and InlC (also called IrpA) is secreted (159, 185, 493). It has been demonstrated that the LRRs and IR of InlA are sufficient to confer an invasive phenotype when expressed on the surface of latex beads or the noninvasive bacteria *L. innocua* or *E. faecalis* (475). Similar findings have recently been made for InlB (83, 83a).

InlA binds to E-cadherin, and it has been shown that this interaction is critical for internalin-mediated invasion of epithelial cell lines (543). It has also been demonstrated the LRR and IR are both necessary and sufficient for this interaction (475). No ligand has been identified for any of the other internalin family members. InlA is one of the few proteins for which a covalent linkage to the cell wall has been demonstrated (474).

The purpose of multiple internalin proteins is unclear, but it has been hypothesized that they confer tropism toward different cell types. For example, it has been suggested that InlB is critical for the invasion of *Listeria* into hepatocytes as well as several other cell types (161, 368). This result is disputed by in vivo experiments in which it was determined that InlB deletion mutants are able to invade hepatocytes (283) but are unable to escape the phagocytic vacuole and replicate intracellularly (284). Indeed, the precise role of any of the internalins in vivo is not yet entirely clear (131).

Staphylococci

Staphylococci are common, highly virulent nosocomial pathogens that are receiving widespread attention due to the appearance of strains that are resistant to all useful antibiotics. Primary to their ability to establish infection in the hospital setting is their ability to bind biomaterial deposited on catheters and other foreign bodies (226). With the exception of protein A, every staphylococcal wall-anchored surface protein thus far characterized is an MSCRAMM. The regulation of surface proteins in *S. aureus* is widely believed to be under the control of the *agr* virulence regulon, which induces the expression of surface proteins during growth at low cell density (384, 591, 665). However, a recent study of the collagen adhesion protein suggests that it is under the control of another, overlapping regulatory system, *sar* (262).

It has been reported that the staphylococcal epidermal cell differentiation inhibitor (EDIN) (763) has a cell wall sorting signal; however, this report appears to be incorrect (215). The C-terminal domain of the protein contains a sequence, LPRGT, which is similar but not identical to the consensus LPXTG motif, and has a tail containing two lysine residues (366). EDIN, however, does not possess a significantly hydrophobic stretch of residues at its C-terminal end. In addition, the protein is found primarily in culture supernatants, and amino acid analysis demonstrates that EDIN is not processed at its C-terminal end, thereby indicating that this protein is not anchored to the cell wall (366, 763).

Protein A. Staphylococcal protein A was the first nonimmune Ig binding protein identified (222). Since its discovery, it has become the best studied and most widely used surface protein of gram-positive bacteria. Early studies established the localization of protein A to the cell wall and even suggested that the protein was covalently attached to the staphylococcal peptidoglycan. The sequence of protein A was determined in 1983 and 1984; however, it should be noted that the earlier publications included a sequencing error at the 3′ end of the gene that eliminated the proper translation of the coding sequence for the cell wall sorting signal (291, 803). The IgG binding properties of protein A are due to the presence of five (in some strains, four) almost identical globular domains that bind the Fc regions of IgG1, IgG2, and IgG4. The three-dimensional structure of a protein A-antibody complex revealed that the protein A-Ig interaction occurs at the C_H2 and C_H3 domains of the Fc fragment (143). Despite all that is known about the structure and binding properties of protein A, almost nothing is known about its exact role in virulence. Studies on protein A mutants in animal model systems have yielded conflicting results, suggesting that other proteins may play a greater role in staphylococcal pathogenesis than protein A (226, 618).

Collagen adhesion protein of *S. aureus*. The gene encoding the collagen adhesion protein of *S. aureus* (*cna*) was cloned from a phage expression library by screening with antibodies raised against a purified collagen adhesion protein (626). Like other MSCRAMMs of other Gram-positive bacteria, the Cna protein has a large unique N-terminal region followed by a set of 187 amino acid repeats, a proline-rich region, and a classic cell wall sorting signal. The collagen-binding domain had been localized to the unique N-terminal domain (622, 625), an observation now verified by the recent determination of the crystal structure (773). The role of the repeat domains, which can vary in number from strain to strain (772), remains unclear.

The fact that *cna* is both necessary and sufficient for the binding of *S. aureus* to cartilage has been demonstrated convincingly in vitro (772). In addition, mutants with mutations in the *cna* gene are less virulent in a rat experimental endocarditis model and in a mouse septic arthritis model (623). However, a recent study of clinical isolates from patients with endocarditis or bone or joint infection found that strains from only half of these patients possessed the *cna* gene, which indicates that collagen binding is probably not a prerequisite for these types of infection (692). The potential of collagen adhesin as a staphylococcal vaccine is being explored (589).

Fibronectin binding proteins of *S. aureus*. *S. aureus* was the first bacterium in which specific binding to fibronectin could be demonstrated, although the gene product responsible remained elusive for almost a decade (462). A >200-kDa fibronectin binding protein was purified from *S. aureus* Newman (191, 233), and soon thereafter a gene encoding a similar activity was cloned from strain 8325-4 (218). Sequencing of this gene, *fnbA*, revealed that it encodes a surface protein, designated FnBPA, with a predicted M_r of 108,000 (727). The gene for another fibronectin binding protein, FnBPB, was discovered only 682 nucleotides downstream of the *fnbA* gene (402), although not all *S. aureus* strains possess both genes (226).

Both FnBPA and FnBPB are similar in their general organization to the FnBPs from other gram-positive cocci such as *Streptococcus pyogenes* (see above). FnBPA is a 982-residue protein composed of a unique N-terminal domain that is interrupted by two copies of a 35-amino-acid repeat, termed B1 and B2 (727). Downstream from the B repeats are 3.5 repeats of 38 amino acids that are homologous to repeat domains found in the other fibronectin binding MSCRAMMs from *S. pyogenes* and *S. dysgalactiae*. FnBPB does not possess B repeats and is only 45% similar to FnBPA in the N-terminal domain, but it is essentially identical to FnBPA in all other aspects (402).

A study of *S. aureus* 8325-4 revealed that binding of *S. aureus* to fibronectin-coated coverslips was not significantly affected in either an *fnbA* or *fnbB* single mutant but was completely abolished in a double mutant. Complementation with either one of the two *fnb* genes restored the fibronectin binding phenotype to near wild-type levels (282), thereby demonstrating that both genes can be expressed in *S. aureus* and that both are capable of mediating staphylococcal adherence to fibronectin. Antibodies to this protein are capable of providing protection against infection in several model animal systems, indicating that this protein may be a potential vaccine candidate (514, 683, 684, 708). The role of this protein in pathogenicity is still the subject some debate, since mutants do not display a virulence defect in a rat model of endocarditis (219).

Fibrinogen binding protein of *S. aureus* (clumping factor). *S. aureus* forms clumps in plasma due to its ability to bind fibrinogen, a multisubunit glycoprotein that is proteolytically converted into fibrin, a major component in blood clots. Fibrinogen is deposited in large quantities at wound sites and is quickly deposited on synthetic material such as catheters (226). Fibrinogen binding, therefore, is seen as a major primary virulence determinant in *S. aureus*, especially in infections due to the presence of foreign bodies.

The fibrinogen binding protein of *S. aureus* was identified by transposon mutagenesis followed by screening for mutants unable to clump in plasma (531). All four mutants isolated had a defect in a gene (*clfA*) encoding an 896-amino-acid protein that harbors an N-terminal leader peptide and a C-terminal cell wall sorting signal. The protein has an N-terminal nonrepetitive domain that is responsible for binding fibrinogen. The clumping-factor protein does not possess a typical proline-rich wall-spanning segment but instead has a very unusual stretch of 154 amino acids composed almost entirely of alternating serine and aspartate residues. This domain is not involved in ligand binding and has recently been shown to be required for the proper display of the molecule on the surface of the cell (308). Mutants with mutations in *clfA* are defective for adherence to fibrinogen-coated surfaces including coated catheter material, suggesting that clumping factor is involved in the establishment of infections due to the presence of foreign bodies (226).

It had been proposed that coagulase, a mostly secreted enzyme found in *S. aureus*, was responsible for the clumping phenotype, since it has been shown to bind fibrinogen in vitro (67). Coagulase mutants, however, were still able to clump in plasma and were unable to cause clotting, whereas *clfA* mutants were unable to clump but were still able to clot plasma (532). These results indicate that the fibrinogen binding clumping factor is the product of the *clfA* gene and not of *coa*. A detailed kinetic study to address the relative contributions of coagulase and clumping factor to the binding of *S. aureus* to fibrinogen-coated surfaces was carried out with a radial-flow chamber (157). By using this method, it was possible to determine the binding constants of intact cells under a variable set of shear force conditions. Clumping factor, not coagulase, was found to be primarily responsible for both the initial attachment and the resulting adhesion.

Fibrinogen binding protein of *S. epidermidis*. Like *S. aureus*, the coagulase-negative bacterium *Staphylococcus epidermidis* is a frequent nosocomial pathogen and has been shown to bind to fibrinogen deposited on synthetic material such as catheters (629). The gene encoding a fibrinogen binding protein from *S. epidermidis* HB has recently been cloned and has been designated *fbe* (590). Although they are not identical in primary sequence, the basic structure and organization of the Fbe protein is almost identical to that of the fibrinogen binding protein from *S. aureus* (see above), including the unusual region of alternating serine and aspartate residues immediately preceding the cell wall sorting signal. The fibrinogen binding site was identified by using a phage display technique, and the domain was mapped to the same region found to be responsible for fibrinogen binding in the *S. aureus* homologue. Strangely, although this protein does bind fibrinogen and is similar to the clumping factor from *S. aureus*, *S. epidermidis* cells do not clump in the presence of plasma (590).

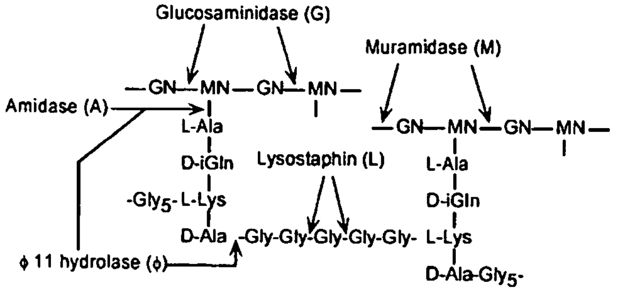


FIG. 17. Sites of hydrolysis of various muralytic enzymes on the peptidoglycan of *S. aureus* (see the text). Modified from reference 575 with permission of the publisher.

TARGETING: BINDING OF POLYPEPTIDES TO THE CELL SURFACE

The protein secretory pathway (Sec) releases polypeptides into the surrounding medium of gram-positive bacteria. Some of these secreted products bind to the bacterial surface of microorganisms and play critical roles in the synthesis and turnover of the peptidoglycan exoskeleton as well as the pathogenesis of animal infections. We describe here what is known about the mechanisms by which secreted proteins are directed to the cell surface and the physiological role these proteins play.

Murein Hydrolases

The peptidoglycan of gram-positive organisms is hydrolyzed at specific times and sites during physiological growth of the exoskeleton (261). This is accomplished by murein hydrolases. These enzymes have been studied extensively, and an excellent review summarizes what is known about their activities and physiological roles (725). Murein hydrolases can be grouped according to their enzymatic activities; the various peptidoglycan cleavage sites of these enzymes are diagrammed in Fig. 17 (250). *N*-Acetylmuramidases (muramidase) and *N*-acetylglucosaminidases (glucosaminidase) cleave MurNAc(β1-4)GlcNAc and GlcNAc(β1-4)MurNAc, respectively (793). Lytic transglycosylases and lysozymes also attack the glycan chains (349); however, these enzymes have not been found in gram-positive bacteria and are not discussed here. Several different classes of enzymes attack the peptide backbone of the cell wall. *N*-Acetylmuramoyl-L-alanine amidase (amidase) cleaves the amide bond between the D-lactyl group of MurNAc and the amino group of L-Ala. Lysostaphin and *Myxobacter* AL1 proteases are glycyl-glycine endopeptidases that cleave the pentaglycine crossbridge of staphylococcal peptidoglycans (189, 709). Another class of enzyme cleaves the attachment site of the peptidoglycan crossbridges: D-Ala–X (250). In staphylococci, the D-Ala is linked to pentaglycine, and this bond is cleaved by the murein hydrolase of phage ϕ11 (LytA) (576). In other bacterial species, the D-Ala can be linked to L-Ala–L-Ala, D-iAsn, or the side chain amino group of *m*-diaminopimelic acid, D-ornithine, and L-Lys (710). Enzymatic activities that

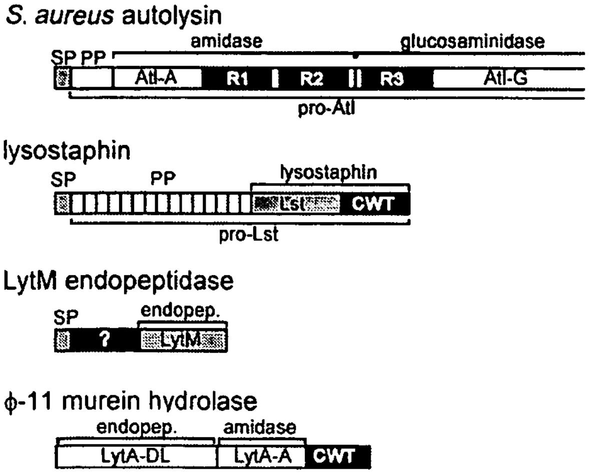


FIG. 18. Domain structure of the peptidoglycan hydrolases of *S. aureus*. Autolysin is synthesized as a preproenzyme, and after cleavage of its N-terminal signal peptide (SP), soluble pro-Atl is released into the extracellular milieu. The repeat domains R1 to R3 function to direct pro-Atl to its receptor on the equatorial surface rings of staphylococci. Proteolytic cleavage generates a propeptide of unknown function (PP), mature amidase with C-terminal R1 and R2 repeats (Atl-A), and glucosaminidase with an N-terminal R3 repeat (Atl-G). Lysostaphin is also synthesized as a preproenzyme, and after signal peptide cleavage, soluble pro-Lst is released into the extracellular milieu. Proteolytic cleavage of 15 tandem repeats of a 13-residue peptide generate mature Lst, which functions as a glycyl-glycine endopeptidase. The C-terminal cell wall-targeting domain (CWT) directs Lst to its receptor on the surface of *S. aureus*. LytM endopeptidase is probably exported by an N-terminal signal peptide. Its C-terminal domain displays some homology to the endopeptidase domain of lysostaphin, whereas the N-terminal domain of LytM is uncharacterized. ϕ11 murein hydrolase is encoded by a lysogenic phage (ϕ11) and exported by a lysis mechanism requiring another phage protein (holein). The enzyme is composed of two domains, one of which is thought to be responsible for amidase activity (LytA-A) and the other is responsible for D-Ala-gly endopeptidase activity (LytA-DL). The C-terminal cell wall-targeting signal of LytA displays homology to that of lysostaphin and also functions to direct this enzyme to its receptor in the cell wall envelope of *S. aureus*.

cleave these peptide bonds of D-Ala have been found; however, the primary structures of these enzymes have hitherto not been identified (250). Carboxypeptidases trim the murein pentapeptide subunits by removing the terminal residue of D-Ala–D-Ala; these membrane-anchored PBPs do not hydrolyze the peptidoglycan and are therefore also not considered here (761).

Sequence alignment of murein hydrolases of gram-positive bacteria revealed that most of these enzymes display a domain structure (404). In general, murein hydrolases harbor an N-terminal signal peptide followed by a second domain containing the enzymatic activity (404). In addition, these proteins harbor repeat structures that flank either the N- or C-terminal side of the enzymatic domain (404). Figure 18 displays the domain structure of murein hydrolases from *S. aureus*. Although the enzymatic domains are often conserved between enzymes from different species, the repeat domains are not. The functions of these repeat domains have been studied most extensively for *Streptococcus pneumoniae* LytA amidase, *Staphylococcus simulans* lysostaphin (Lst), and *S. aureus* Atl and are discussed in detail below. Ghuysen et al. reported sequence and structural similarity between the noncatalytic regions of muramidases and amidases from *Clostridium acetobutylicum* and *Bacillus* spp. and the crystallized structure of *Sreptomyces albus* G Zn DD-peptidase (253). This region is required for the binding of DD-peptidase to insoluble peptidoglycan, suggesting that murein hydrolases from several other gram-positive bacteria retain this domain to bind to their substrate (253). This substrate binding domain is distinct from targeting domains, which direct some murein hydrolases to specific sites on the bacterial cell surface (see below).

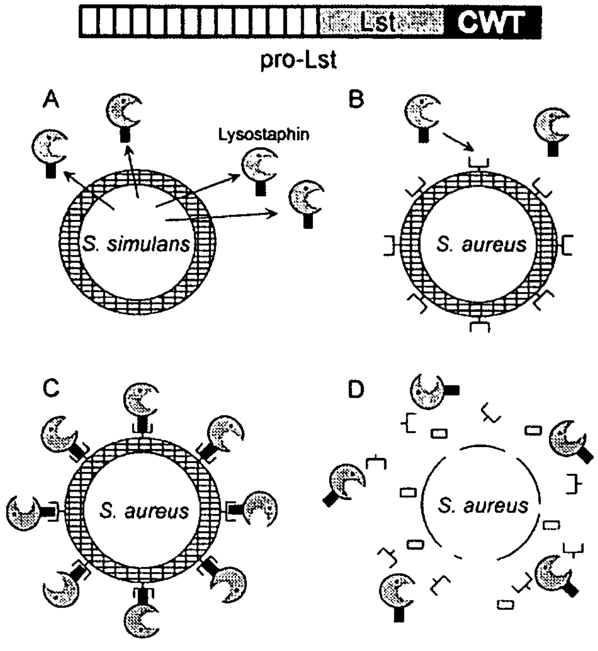


FIG. 19. Targeting of lysostaphin to the cell wall envelope of *S. aureus*. (A and B) After secretion by *S. simulans* bv. staphylolyticus (A), prolysostaphin is converted to the active mature species and targeted to those staphylococci that harbor the appropriate receptor on the bacterial surface (B). (C and D) Mature lysostaphin is a glycyl-glycine endopeptidase that cleaves the peptidoglycan crossbridge of staphylococci (C), thereby hydrolyzing the cell wall envelope and lysing the bacteria (D).

Staphylococcus simulans Lysostaphin

Staphylococcus simulans bv. staphylocolyticus secretes lysostaphin (709), which hydrolyzes the peptidoglycan of all the staphylococci that synthesize peptidoglycan with pentaglycine crossbridges (95, 878) (Fig. 19). In this way, lysostaphin functions as a bacteriocin to kill competing microorganisms in mixed bacterial populations. Lysostaphin is synthesized as a preproenzyme and initiated into the secretory pathway by an N-terminal leader peptide. The proenzyme is released into the culture medium and contains 15 tandem repeats of a 13-residue peptide at the N-terminal end (324). Prolysostaphin is 4.5-fold less active than mature lysostaphin, and the N-terminal repeats are removed in a growth phase-dependent manner by a secreted cysteine protease (782). Mature lysostaphin cleaves the peptidoglycan of *S. aureus* cells much more actively than it cleaves the peptidoglycan of its *S. simulans* host (314). There appear to be two reasons for this. First, *S. simulans* cells elaborate an immunity factor that causes the incorporation of serine residues into the cell wall crossbridge (782). Although, on balance, only one or two serine residues are found in the otherwise poly-glycyl crossbridge, this modification renders *S. simulans* resistant (immune) to this bacteriocin (782). The *S. simulans* gene required for incorporation of the serine residues, *lif* (lysostaphin immunity factor), displays striking homology to the *femA* and *femB* genes (145, 331, 513). The latter genes are thought to specify enzymes which synthesize the cell wall crossbridge by using lipid II peptidoglycan precursor and charged Gly-tRNA as substrates (43). Consistent with the notion that

Lif is the functional equivalent of a FemA/FemB enzyme is the observation that Lif can complement *femB* mutant staphylococci (798). Furthermore, immediately adjacent to the immunity factor gene is a locus encoding seryl-tRNA, suggesting that Lif may also utilize charged tRNAs as substrates (782). Recently, a glycyl-glycine endopeptidase of *Staphylococcus capitis* was described and characterized (764, 765). The molecular structure, targeting mechanism, and immunity of this enzyme appear to be identical to those described for lysostaphin.

Purified lysostaphin binds much more avidly to *S. aureus* than to *S. simulans* cells (21, 519). When added to mixed bacterial populations, purified lysostaphin kills 1,000 *S. aureus* cells for every *S. simulans* cell (21). This selectivity requires the C-terminal targeting domain of Lst, which is located in the 92 C-terminal amino acids (21). When deleted from lysostaphin, this mutant enzyme no longer binds to staphylococci and has lost the ability to distinguish between *S. aureus* and *S. simulans* cells (21). Furthermore, when the targeting domain is linked to the C terminus of secreted enterotoxin B, the fusion protein is exported but directed to the cell wall compartment of *S. aureus* cells. Similarly, when fused to the C terminus of reporter proteins such as glutathione *S*-transferase, the recombinant protein binds to the surface of *S. aureus* cells but not of *S. simulans* cells (21). Hence, lysostaphin is specifically directed to the surface of *S. aureus* by its C-terminal targeting domain (20). This targeting mechanism functions in mixed bacterial populations and therefore must be species specific. In other words, murein hydrolases such as lysostaphin are probably designed such that the targeting domain recognizes receptors found on the surface of specific species of microorganisms. It follows that the targeting domain cannot recognize peptidoglycan alone, because its chemical structure is largely conserved in all gram-positive bacteria (20).

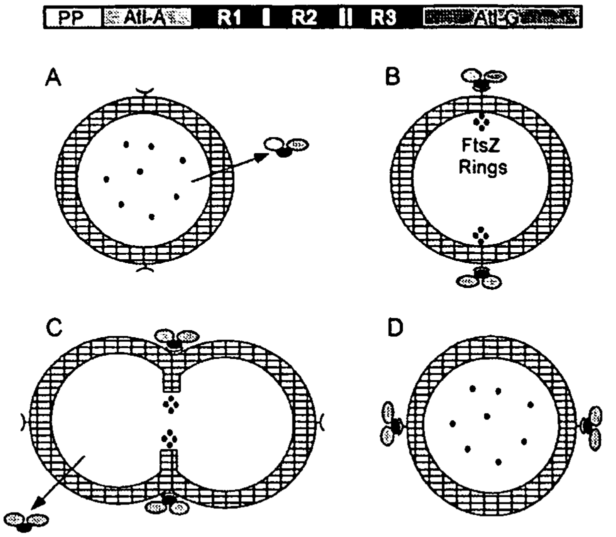


FIG. 20. Targeting of Atl to the equatorial surface rings of *S. aureus*. Autolysin is exported from the bacterial cytoplasm (A), and extracellular pro-Atl is targeted to receptor molecules representing the equatorial surface rings of staphylococci that mark the future cell division site (B). Targeting requires the repeats domains (R1 to R3) or pro-Atl. Proteolytic cleavage of pro-Atl generates mature amidase (Atl-A) and glucosaminidase (Atl-G), each of which retains one or two repeat domains and remains bound to the surface rings. The enzymes hydrolyze the cell wall at the cell division site, an event that is probably synchronized with intracellular contraction of FtsZ rings (C), thereby generating two fully separated daughter cells (D). In staphylococci, division occurs perpendicular to the previous cell division plane and targeting of pro-Atl to the second equatorial surface ring can initiate hydrolysis at the future cell division site.

Staphylococcal Autolysin

Not all murein hydrolases function to kill microorganisms. Staphylococci divide in a direction perpendicular to the previous cell division plane (258, 259, 261). These coccal cells require hydrolysis of their thick peptidoglycan layer at the midcell to synthesize new cell wall and separate the dividing daughter cells (261). This depends on the Atl enzyme, because *atl* mutants show characteristic defects in cell separation and grow as large clusters of staphylococci (606, 766). The *atl* gene specifies a large polypeptide (preproenzyme) with several domains (225, 605). An N-terminal signal peptide is required for export (22). Secreted Pro-Atl is processed at two sites, residues 198 and 775 (22, 605). These cleavage events generate an N-terminal propeptide of unknown function as well as two domains with enzymatic functions, amidase and glucosaminidase (732, 786). Three repeat domains are located at the center of pro-Atl, such that mature amidase retains two C-terminal repeats and mature glucosaminidase retains one N-terminal repeat domain (605). The repeat domains function to direct Atl to the future cell division site of staphylococci (22), a structure named the equatorial surface ring (766, 767, 864) (Fig. 20). Targeting of pro-Atl occurs before it is processed; however, mature amidase and glucosaminidase remain bound at the surface ring structure (22, 864). The significance of the timing of processing and targeting is thus far not understood. Fusion of the repeat domains to reporter proteins directs the hybrid proteins to the equatorial surface rings, demonstrating that the repeat domains of Atl properly address the enzyme to the cell division site (22). This mechanism implies that a specific receptor for Atl might be located at this site to attract the secreted enzyme for localized hydrolysis (22). Although this hypothesis provides a model for cell separation in gram-positive bacteria, several questions are left unanswered. To define a spatial location on the cell surface, staphylococci presumably synthesize a specific decoration of the peptidoglycan. This decoration must be positioned in a manner that defines both the equatorial surface ring and future cell division sites. The same dilemma exists in the bacterial cytoplasm, where cell division occurs via constriction of an FtsZ ring structure which is assembled at midcell from soluble components and then constricts to fuse the membrane, thereby separating two daughter cells (54, 505). It is conceivable that the definition of the cell division site is provided by the synthesis of a chemical decoration in the rigid exoskeleton, which might provide an anchoring point for cell wall hydrolases and the membrane fusion machinery alike (681, 682).

As mentioned previously, coagulase-negative staphylococci, such as *S. epidermidis*, are important nosocomial pathogens that adhere to foreign bodies implanted into human tissues (629). Adherence of staphylococci to catheters and other materials is followed by the production of large amounts of biofilm (629). Götz and coworkers screened a collection of Tn*917* insertional mutants of *S. epidermidis* for their inability to produce biofilm and/or adhere to polystyrene surfaces (321). Wild-type staphylococci synthesize a linear carbohydrate polymer composed of β1-6-linked 2-deoxy-2-amino-D-glucopyranosyl, in which almost all moieties are N-acetylated, named polysaccharide intercellular adhesin (PIA) (506, 507). One class of Tn*917* mutants is defective in the production of PIA, and the mutation maps to the *icaABC* operon (323, 508). The *ica* genes specify the enzymatic machine that synthesizes PIA in both mutant *S. epidermidis* and *S. carnosus*, a microorganism otherwise unable to synthesize biofilm (323). A second class of mutants is unable to adhere to polystyrene surfaces, and its Tn*917* insertion maps to the autolysin gene of *S. epidermidis* (*atlE*) (322). Binding of staphylococci to foreign materials is thought to be aided by human plasma proteins. A search for bound plasma proteins reveals that vitronectin may bind to AtlE (322). The authors observed less vitronectin binding to mature Atl-amidase and Atl-glucosaminidase than to pro-Atl, suggesting that the propeptide may be required for binding (322). The autolysin of *S. saprophyticus* is thought to be involved in binding to fibronectin proteins (325). This microorganism causes a large number of female urinary tract infections and expresses a surface protein which binds to fibronectin (246). A search for fibronectin binding activity of cloned staphylococcal sequences revealed the *aas* gene, which is homologous to the *atlE* and *atl* genes (325). Mutant *S. saprophyticus* strains carrying a defective *aas* gene were unable to bind either fibronectin or erythrocytes, and the fibronectin binding activity of purified Aas protein was mapped to the R1 to R3 repeats (325). Thus, the autolysin of staphylococci appears to be a multifunctional protein, required for cell wall hydrolysis at a designated site as well as for attachment of bacteria to eukaryotic proteins and/or mammalian tissues.

Phage-Encoded Murein Hydrolases

Bacteriophage lambda of *E. coli* employs the S lysis protein (holin) to disrupt the host cell membrane (11, 63, 245, 664). This mechanism allows phage-encoded intrabacterial murein hydrolases to leave the cytoplasm and degrade the peptidoglycan sacculus, which is located in the periplasmic space of gram-negative bacteria (61, 494). Without murein hydrolysis, phage particles could not be released into the extracellular medium (664). A similar strategy is used by the bacteriophages of gram-positive organisms (679). For example, *S. aureus* phage φ11 expresses a murein hydrolase (LytA) devoid of an N-terminal signal peptide (834, 835). The LytA protein has a C-terminal targeting signal similar to that of lysostaphin (21), and its N-terminal domains code for both its D-Ala–Gly endopeptidase and amidase activities (576). Adjacent to the *lytA* gene is an open reading frame specifying a lysis protein with sequence homology to the holins of bacteriophages of gram-negative bacteria (70). A similar genetic arrangement to that of φ11 has been observed for staphylococcal phages 80α and Twort (68, 498). Thus, although this has never been demonstrated directly, it seems likely that phage-encoded murein hydrolases are released from the cytoplasm of gram-positive bacteria only after the holin-induced disruption of the cytoplasmic membrane that occurs during the phage lytic cycle.

Several groups have searched for autolysins, enzymes that degrade the cell wall of gram-positive bacteria at the end of logarithmic growth. Autolysin activity can be measured on agar plates containing heat-killed bacteria (274, 606). Mutant colonies that fail to hydrolyze peptidoglycan can be screened from a population of transposon insertion mutants. Several such mutants were isolated in *S. aureus*, and mutations in *lytA*, *atl*, *lytM*, and *lytRS* were found in screen (98, 605, 660, 835). It now appears that two of these genes encode staphylococcal autolysins. The Atl enzyme, specifying amidase as well as glucosaminidase activity, is described above. The *lytM* gene encodes a protein harboring an N-terminal signal peptide followed by an enzymatic domain that is homologous to that of lysostaphin and probably functions as a glycyl-glycine endopeptidase (660). LytM does not harbor any of the known targeting signals of either lysostaphin or Atl, and its location on the staphylococcal surface is still unknown. The *lytA* gene is encoded by bacteriophage φ11, which has lysogenized strain 8325 (834). The *lytRS* genes display homology to a two-component regulatory system involved in signal transduction (98). The *lytRS* locus controls the activity of two other genes, *lrgA* and *lrgB*, encoding a staphylococcal holin lysis protein (LrgA) similar to those described for phages φ11, 80α, and Twort and a protein with unknown function (LrgB) (99).

Murein hydrolases of gram-positive bacteria appear to fall into two classes: the enzymes which are encoded by genes on the bacterial chromosome, exported by an N-terminal signal peptide, and presumably involved in physiological cell wall turnover (class I), and others that are located on the chromosome of bacteriophages, devoid of signal peptides, and exported by a general lysis mechanism (class II). The latter class of genes found in the screen described above were identified due to the induction of lysogenic phages at the end of logarithmic growth.

S. pneumoniae

S. pneumoniae synthesizes a major autolysin which hydrolyzes the cell walls of these bacteria in stationary-phase cultures (795). The enzyme is thought to exist in two states, a cytoplasmic inactive species and a membrane-associated active counterpart (91). The inactive species (E) accumulates in cells that are grown in ethanolamine, whereas the more active enzyme (C) accumulates in cultures grown in media containing choline (91). The inactive enzyme has been purified to homogeneity by affinity chromatography on choline-Sepharose (91) or by DEAE ion-exchange chromatography (702). The pure enzyme can be activated by the addition of choline (352), suggesting that choline is a necessary cofactor (265). Presumably, LytA undergoes a conformational change when encountering choline, resulting in enzymatic activity (350–352, 698). Cloning and sequencing of the *lytA* gene revealed an open reading frame specifying a 31-kDa polypeptide (242). The open reading frame displays sequence homology to the gene sequence encoding enzymatic domains of many other known amidases (677). However, six repeat domains located at the C-terminal end of LytA (242, 699) are not found in the amidase sequences from other bacterial species but are present in a variety of phage-encoded murein hydrolases of *S. pneumoniae* (499). When expressed in *E. coli* and purified to homogeneity, these LytA repeats were shown to bind choline and hence have been named choline binding repeats. Thus, LytA binds to choline-containing teichoic acids, resulting in its enzymatic activation and peptidoglycan hydrolysis on the cell surface (350–352). The common repeat structure of pneumococcal cell wall teichoic acid and lipoteichoic acid (31, 207, 211, 379) [Glc(β1-3)AATGal(α1-4)GalNac(6-Cho-P)(α1-3)GalNac(6-Cho-P)(β1-1)ribitol-P] suggests that LytA can bind to both structures. The *lytA* open reading frame did not reveal an N-terminal signal peptide (242), and either LytA is not exported under physiological conditions or its export may occur by a pathway other than the known Sec machinery (156). When expressed in *E. coli*, LytA appears to be translocated across the cytoplasmic membrane and was detected by immunogold labeling on the periplasmic side of the cytoplasmic membrane (156). Immunogold labeling of *S. pneumoniae* revealed LytA staining on the cell surface, indicating that the enzyme is also translocated and surface displayed (156). Future work is needed to further characterize the export mechanism of LytA.

Briles et al. identified PspA (pneumococcal surface protein A) as a major antigen during animal infections by *S. pneumoniae* (93). Antibodies raised against purified PspA protect mice from challenge with virulent strains (92) and pneumococcal *pspA* mutants are less virulent in a mouse model system

(530). PspA displays size and antigenic variation; however, the immunodominant antibodies are opsonic (phagocytic) and cross-reactive with PspA species of other strains (528, 529, 659, 779). Thus, PspA might serve as a vaccine candidate in protecting animals from pneumococcal infections. Sequencing of the structural gene revealed that PspA is synthesized with an N-terminal signal peptide (875). Because much of the N-terminal part of the mature polypeptide showed a 7-residue periodicity of hydrophilic and hydrophobic residues, it is likely that this domain of PspA assumes a coiled-coil structure. At the C terminus, PspA contains repeat domains homologous to those of LytA (875). Shortening of the C-terminal domain to five repeats abolished surface display and released the mutant protein into the culture supernatant (876). These results are comparable to those obtained for LytA, which showed that binding to the pneumococcal surface required as many as four repeat domains (243, 699). The LytA repeat domains are not only required but also sufficient for binding to choline-containing teichoic acids. This has been demonstrated both by expressing fusion proteins with LytA repeats in pneumococci (676, 746) and by adding purified recombinant proteins to cultures of *S. pneumoniae* (135).

Bacillus

Bacillus and *Clostridium* are spore-forming species. During sporulation, the mother cell undergoes asymmetric cell division to engulf and nurture the spore (501). The cell wall peptidoglycan and associated structures (cortex) of the spore are distinct from that of cells growing under vegetative conditions (646, 836, 852). During germination, the mother and spore peptidoglycan are dissolved and the released spore once again grows as a vegetative cell (101). Sequencing of the *B. subtilis* genome revealed at least 16 genes with homologies to known murein hydrolases, most of which are amidases (454). To characterize these enzymes, several investigators have used zymography, i.e., the activity measurement of renatured proteins after their separation on SDS-PAGE by overlaying with heat-killed bacteria (748). Murein hydrolase activity can be visualized as a zone of bacteriolysis representing a protein with unique mobility. When this experiment is performed with proteins of *B. subtilis* cell extracts, at least 10 proteins with bacteriolytic activity can be revealed (224), suggesting that most if not all of the 16 genes specifying murein hydrolases are indeed expressed. Clues to the function of these genes came from the generation of specific knockout mutations. The two most prominent zymographic species, a 90-kDa glucosaminidase (LytD) and a 50-kDa amidase (LytC), may not function as autolysins, because strains carrying knockout mutations in their structural genes revealed either no (*lytD*) or little (*lytC*) decreased autolysis of stationary-phase cultures (460, 516, 517). No effects on either cell separation, sporulation, or germination of this organism were observed. The *lytC* gene is located within the *lytRABC* operon specifying the regulatory transcription factor LytR, lipoprotein LytA, and LytB, the modifier protein for the LytC amidase (473). LytB binds to LytC, altering its enzymatic activity from a random to a processive amidase removal of peptide substituents along single glycan strands (332, 333). LytB is similar in sequence to SpoIID (458, 473), a factor required for dissolution of the forespore septum with the mother cell (364). Transcription of *lytRABC* is regulated in part by the flagellar transcription factor sigma D (327), which also controls all *lytD* expression (459, 473). Because *sigD* mutants grow as long filaments, it is suspected that this transcription factor may control the expression of murein hydrolases required for cell division (328). There appears to be at least one enzyme other than *lytC* involved in this, because *lytC* mutants do not display filamentation (748). With the completion of the *B. subtilis* genome sequence, it is presumed that all open reading frames specifying murein hydrolases have been identified. Little is known, however, about the targeting of any of these enzymes. The SpoIID protein displays sequence homology to the modifier protein (LytB) of the LytC amidase (458). Whether this homologous domain is involved in directing these proteins to the septum is still unknown. Mother cell lysis depends on the compensatory effect of the LytC (CwlB) and CwlC hydrolases, suggesting that these two enzymes have redundant functions (457). The CwlD hydrolase is thought to be required for spore cortex maturation (19, 645, 719). Germination of spores also requires murein hydrolases, and several enzymes appear to fulfill compensatory functions, for example CwlJ and SleB (369, 558, 559). Whether any of these *Bacillus* enzymes are directed to their subcellular location by a specific targeting event is also still unknown.

Clostridium

Clostridium difficile, the causative agent of pseudomembranous colitis and antibiotic-associated colitis in humans, releases two toxins, TcdA and TcdB, which are thought to be major virulence factors of this organism (406, 408, 424). Both toxins are targeted into the cytosol of eukaryotic cells, where they function as glucosidases to modify small GTP binding proteins such as Rho, Rac, and Cdc42 by using UDP-Glc as a cosubstrate (10, 815). Glucosidation leads to inactivation of GTPase activity and of the signaling function of these molecules, causing actin rearrangements, fluid loss, and, finally, cell death (407). Injection of glucosylated Rho into eukaryotic cells has the same cytotoxic effect as injection of TcdB, indicating that Rho is the main target of toxin action and is dominant over unmodified Rho (407). *C. sordellii* and *C. novyi* toxins also glucosylate small GTP binding proteins, although the alpha-toxin of *C. novyi* employs UDP-GlcNAc as a substrate for modification (647, 722).

Neither TcdA, TcdB, nor any other member of a family of large clostridial toxins is synthesized with an N-terminal signal peptide (815). Although the mechanism by which these toxins are secreted and/or released from the bacteria is unknown, once in the extracellular milieu, toxins display binding affinity for mammalian cells and hence are taken up into the eukaryotic cytosol (705). Clostridial toxins A and B are very large polypeptides (308 and 270 kDa) (29, 706), composed of an N-terminal domain containing glucosyl transferase activity (342, 651) and a C-terminal domain with tandem repeats of a 30-residue peptide (705). The repeat domains are responsible for binding to carbohydrate compounds, and TcdA recognizes Galα1-3Galβ1-4GlcNAc (450). Such carbohydrate structures are displayed on the surface of animal cells, accounting for the hemagglutination of rabbit erythrocytes by TcdA (800). Although similar in sequence, TcdB does not hemagglutinate the same cells, presumably because its repeats do not recognize the same carbohydrates (800). Antibodies directed against the C-terminal repeats of TcdA prevent toxicity in a mouse model system, as does preincubation of cells with recombinant proteins carrying the C-terminal repeat domains but not the N-terminal domain with enzymatic activity (705). Thus, the C-terminal repeat domains function as a targeting device that directs clostridial toxins to certain eukaryotic cells.

Several authors observed that the repeat elements found in clostridial toxins have some sequence similarity to the repeat domains of streptococcal glucan binding proteins as well as the LytA repeats of pneumococcal proteins (816, 817, 860). This suggested that the ability to bind carbohydrate elements may be a common property of these repeat structures. In keeping with this hypothesis, aromatic amino acid residues (W, Y, and F) are found regularly in the repeat structures of proteins of gram-positive bacteria with targeting elements, and they may serve as stacking devices for the interaction with carbohydrate ring structures, as has been observed for sugar binding proteins in the periplasm of gram-negative bacteria (860).

Listeria

InlB is a member of the internalin family of bacterial invasins and promotes the entry of *Listeria* into hepatocytes and several other cell lines (161, 163, 368). The broad tropism apparently conferred by InlB might indicate that the InlB receptor is more widespread than E-cadherin, the receptor for InlA. During the infection of mice, *Listeria* has been observed in the liver, and InlB is thought to be essential for multiplication in this organ (130, 161). InlB is synthesized as a precursor bearing an N-terminal signal peptide (235). The N-terminal domain of mature InlB displays sequence homology to other members of the internalin family whereas the C-terminal 232 amino acids are homologous to the targeting domain of a *Listeria* amidase (82). When added externally to *Listeria*, purified InlB binds to the bacterial surface (82). Binding of InlB is dependent on the C-terminal targeting signal, since mutant proteins without this sequence are found in the extracellular medium (82). The addition of purified InlB to other bacteria or to latex beads also promotes uptake of these organisms or particles into hepatocytes, indicating that InlB alone is sufficient to promote this function (83). It is not clear why *Listeria* chooses to display InlB via a cell wall-targeting mechanism whereas all other internalins are apparently linked (sorted) to the bacterial peptidoglycan. To investigate this further, Cossart and coworkers designed a recombinant InlA protein which was displayed on the listerial surface via the C-terminal targeting signal of InlB (82). This hybrid protein was functional in promoting invasion in a manner indistinguishable from wild-type InlA (162, 164). It is conceivable that *Listeria* regulates the surface display of its different internalins (162) and that this regulation is, at least in part, carried out by their anchoring and release from the cell surface.

Listeria p60 is required for cell growth and intestinal invasion of this organism (334, 861). p60 homologs have been found in many listerial species (100). The N- and C-terminal domains of p60 species are conserved in all *Listeria* species; however, the central sequences are variable (100). Cytotoxic T cells that recognize p60 epitopes are protective against *Listeria* infections in a mouse model system, suggesting that this surface protein presents an immunodominant antigen (74, 247). Purified p60 displays murein hydrolase activity, and overexpression of p60 leads to the appearance of significantly shortened *Listeria* cells. The C-terminal domain of p60 displays homology to the repeat domains of an autolysin of *Enterococcus faecium* (234), suggesting that p60 may be targeted to the cell surface and required for peptidoglycan hydrolysis similar to the staphylococcal Atl enzyme.

Streptococci

Pancholi and Fischetti described the purification of several different glycolytic enzymes from *S. pyogenes* cell extracts (614). SDH (glyceraldehyde-3-phosphate dehydrogenase) and enolase were isolated from cell wall extracts obtained after enzymatic peptidoglycan hydrolysis and removal of the resulting protoplasts by sedimentation (610). Immunoelectron microscopy, reactivity of affinity-purified antibodies, and enzyme assays with intact streptococci have each indicated that these glycolytic enzymes are found in two locations, the bacterial cytoplasm and the streptococcal surface (610). Surface-exposed and cytoplasmic enzyme appear to be one and the same species, synthesized from a single gene and devoid of an N-terminal signal peptide. Clearly, these glycolytic enzymes are not exported, sorted, or targeted to the envelope of this gram-positive bacterium by any of the established pathways. It will be interesting to see by which mechanism streptococci can display enzymes in two different locations. SDH binds to a variety of eukaryotic proteins (fibronectin, lysozyme, myosin, and actin) (615). Furthermore, purified SDH activates eukaryotic protein tyrosine kinase and protein kinase C by ADP-ribosylation (611). Inhibitors of protein kinase activity prevented *S. pyogenes* invasion of pharyngeal cells, suggesting that host protein phosphorylation plays a role in the uptake of this pathogen (615). Streptococcal enolase binds to plasmin and plasminogen (610). The precise role of this binding in streptococcal invasion and disease is not yet clear. Others have reported that high-affinity plasminogen binding to the surface of *S. pyogenes* is caused by certain M-protein-like proteins (see above) (858).

S. mutans is one of the principal etiologic agents of dental caries (497). This pathogen synthesizes both water-soluble α(1-6) glucan and insoluble α(1-3) glucan by hydrolyzing dietary sucrose (311) and polymerizing the resulting monomeric glucose residues (455). Both types of glucan are components of dental plaque, and the water-insoluble species mediates the adherence of *S. mutans* to smooth dental surfaces (299). Synthesis of glucan is stimulated by the GtfA enzyme, which acts as a sucrose phosphorylase to synthesize Glc-1P (200, 690). The Gtf-I (*gtfB* and *gtfC*) (301) and Gtf-S (*gtfD*) (301) enzymes are responsible for synthesizing water-insoluble and water-soluble glucans, respectively, and streptococcal strains carrying mutations in any of these genes display a defect in the pathogenesis of dental caries in a pathogen-free rat model (865). Sequence analysis of the *gtf* genes and their encoded polypeptides revealed that each of these enzymes is exported by an N-terminal signal peptide. A large N-terminal enzymatic domain is followed by a C-terminal domain with tandem repeats of 20 to 48 amino acids (26, 199, 263, 653, 859). Successive truncations of the C-terminal repeats yielded enzymes that were still active but had impaired binding activity for α1-6-linked glucans, the substrates for their glucosyltransferase activity (199). Thus, the C-terminal repeat domains of Gtf enzymes target these enzymes to their glucan substrates, which are deposited on the dental surface. As mentioned above, the repeat sequences are homologous to those found in large clostridial toxins and pneumococcal choline binding domains (817).

Targeting of S-Layer Subunits

S-layers are two-dimensional proteinaceous crystalline arrays formed by proteinaceous subunits that cover the outer surface of many different kinds of unicellular organisms (53, 741, 742). S-layers have been identified in a range of species from the *Archaea*, *Bacteria*, and *Eucarya* and diverge greatly in their structures and functions. We make no attempt here to cover the many aspects of the hundreds of S-layers that have been identified and will instead focus on what is known about the mechanisms by which these S-layers are targeted to and assembled on the cell surface of gram-positive bacteria. For a more extensive review of what is known about the biology of S-layers, the reader is directed to a series of recent reviews (24, 53, 547, 657, 739).

S-layers are formed by the entropy-driven aggregation of monomer subunits which, in most cases, are exported from the cytoplasm via the general secretory pathway by an N-terminal leader peptide. S-layers are not covalently attached to the cell surface and can usually be extracted either as sheets (30) or as individual subunits in the presence of dissociating agents such as 5 M lithium chloride (500) or EDTA (768) or chaotropic denaturants such as guanidine hydrochloride or urea (357, 740, 743). In many cases the monomeric subunits can spontaneously reassemble into a two-dimensional lattice once the dissociating agent is removed. Lattices formed in vitro usually bear the same hallmark geometric crystal pattern of the original cell-bound S-layer and are often capable of reassociation with the bacterial cell surface. The spontaneous nature by which S-layers assemble means that the primary energetic cost to the cell of manufacturing the S-layer stems from synthesizing the enormous number of subunits necessary to cover the entire cell surface. S-layer proteins are often the most abundant species in cells, comprising up to 15% of the total cellular protein (742). This is not surprising considering that approximately 5×10^5 subunits are required to cover an average-sized cell (741).

S-layers are usually surface exposed, although in at least one strain of *Bacillus anthracis* the S-layer can coexist with a surface-exposed capsule and it appears that the expression and display of one does not interfere with that of the other (545). Multiple S-layers can be simultaneously expressed on a single cell, and it is believed that in some cases one S-layer can serve as the primary attachment or crystallization surface for other layers (418, 428, 546, 799). The S-layer in *Bacillus stearothermophilus* is the envelope component responsible for the attachment of a surface-displayed amylase (175, 177), indicating that these layers can serve as targeting ligands for other proteins.

SLH domains. Only recently has any insight been gained into the manner by which S-layers are attached to the gram-positive cell surface. Recent structural studies have revealed that several S-layer subunits share a region of homology in a domain that has been shown to interact with the cell wall (481, 504, 599). These surface layer homology (SLH) domains have been identified in S-layer subunit protein sequences from several gram-positive species as well as in some other types of surface proteins including the cellulosome of *Clostridium thermocellum* (477). All SLH domains identified thus far have been found at the very beginning or end of the mature proteins (503). An SLH domain is usually composed of either a single or three repeating SLH motifs of approximately 50 to 60 residues (503); a comparison of these SLH motifs reveals that they are fairly divergent with an average identity of 27% and only one universally conserved residue (504).

SLH domains have been speculated to interact either directly with peptidoglycan (599) or with other wall-associated polymers. Recent studies of three different S-layer proteins from different strains of *B. stearothermophilus* have identified two apparently unique secondary wall polymers as the surface ligands responsible for the adherence of the S-layer to the cell surface (176, 670). The SLH receptor on the surface of PV72/p2 was isolated by extracting isolated cell walls with hydrofluoric acid (670). Chemical analysis determined that the receptor molecule was a polymer composed of GlcNAc and ManNAc in a molar ratio of 3.5:1 and with an approximate molecular mass of 20,000 Da (670).

An S-layer subunit from *Thermus thermophilus* containing a single SLH motif seems to bind directly to the peptidoglycan (599). Although *T. thermophilus* is not taxonomically classified as a gram-positive bacterium, it is known to have a similar subcellular architecture. Chemical analysis of the envelope from this bacteria has indicated that the peptidoglycan does not contain any associated secondary wall polymers (656). It therefore appears likely that SLH domains have evolved the ability to recognize a wide variety of surface ligands, including, in some cases, the peptidoglycan itself. This property is perhaps reflected in the loose sequence conservation found among the various SLH domains.

Other S-layer-targeting domains. It is important to note that S-layer proteins from several gram-positive bacteria do not have SLH domains, demonstrating that different adherence mechanisms have evolved for different S-layers. The PS2 protein of *Corynebacterium glutamicum* contains a C-terminal hydrophobic domain of approximately 79 residues that can be removed by protease treatment, resulting in an intact S-layer sheet that is not able to adhere to the *Corynebacterium* cell surface (114, 115). More recently, a secondary wall polymer ligand has been partially characterized for the S-layer proteins from *B. stearothermophilus* ATCC 12980 and the PV72 variant, PV72/p6 (176), both of which lack SLH domains. The composition of this wall polymer appears to differ significantly from that of the ligand for the PV72/p2 S-layer protein; the polymer appears to be composed of glucose and glucosamine in roughly equal ratio (176). It is almost certain that other adherence mechanisms will become apparent as S-layers from more gram-positive bacteria are studied in the future.

CONCLUSIONS AND FUTURE DIRECTIONS

We have summarized here the currently known surface proteins of gram-positive bacteria and the mechanisms of their anchoring to the cell wall envelope. The completion of several microbial genome sequences will soon provide us with a more comprehensive view of the number and nature of these molecules. If this information is combined with biochemical analysis, our understanding of surface protein function will probably expand rapidly. Nevertheless, future work must also further investigate the mechanisms of surface protein anchoring to the cell wall envelope. The cell wall sacculus provides rigid exoskeletal functions for microorganisms and requires proper targeting of surface proteins for its concerted assembly and turnover. A detailed knowledge of these mechanisms appears absolutely necessary to our understanding other physiological events such as bacterial cell division and separation. We apologize to all those authors whose work was not mentioned here either because of space constraints or because of our oversight.

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